REMARKS

Claims 9, 11 and 14 presently appear in this case. No claims have been allowed. The Official Action of February 21, 2006, has now been carefully studied. Reconsideration and allowance are hereby respectfully urged.

Briefly, the present invention is directed to a method for inducing myelination and remyelination of neurons by administering to a patient in need thereof an effective amount of IL6R-IL6 chimera.

Claims 9-12 and 14-15 have been rejected under 35 USC 112, first paragraph. The examiner concedes that the specification is enabling for a method of inducing myelination and remyelination of Schwann neurons by providing a chimera of IL6R-IL6 but states that it is not enabling for a method of inducing myelination and/or remyelination of unknown populations of neurons. This part of the rejection is respectfully traversed.

Claim 9 has now been amended to specify that the method is for inducing myelination and remyelination of neurons. In order to expedite allowance, reference to the utilities of protecting neurons from NMDA-induced cell death, from neurotoxicity induced by excitatory amino acids, and from

toxicity caused by withdrawal of NGF, have been removed from the claims without prejudice toward continuation of prosecution thereof in a continuing application. As to the breadth of inducing myelination and remyelination, the examiner states that the specification is only enabling for myelination and remyelination of Schwann neurons. However, additional experimentation has now been conducted and published showing that, not only does the invention work for peripheral neurons such as Schwann cells, but also CNS neurons such as oligodendrocytes. Attached hereto are two publication from the laboratory of the present inventors: Zhang et al, "Enhancement of Oligodendrocyte Differentiation from Murine Embryonic Stem Cells by an Activator of gp130 Signaling" *Stem Cells* 22:344-354 (2004) and Zhang et al, "Increased myelinating capacity of embryonic stem cell derived oligodendrocyte precursors after treatment by interleukin-6/soluble interleukin-6 receptor fusion protein" *Mol. Cell. Neurosci.* 31:387-98 (2006) (Epub 2005 Dec 1). In light of these publications, it is not unbelievable that the present invention is applicable to inducing myelination and remyelination of neurons generally, as alleged in the present specification, and not just in the Schwann neurons of the examples that were in the application as filed.

Reconsideration and withdrawal of this part of the rejection is respectfully urged.

The examiner states that while the instant specification discloses *in vitro* experiments, it does not provide disclosure for the treatment of patients suffering from traumatic nerve degeneration or suffering from a demyelinating disease of the CNS or PNS, Alzheimer's disease, Parkinson's disease, ALS or MS. The examiner states that the specification fails to teach how the administration of IL6R/IL6 chimera would effectively treat such diverse disorders. The examiner conceded that the specification discloses an *in vivo* murine model of chronic relapsing MS, but states that it does not disclose the result of the disclosed regimen. This part of the rejection is respectfully traversed.

In order to expedite allowance of this case, claims 10 and 15 have now been deleted. As to claim 11, it is urged that in view of the fact that it has been shown that IL6R/IL6 chimera induces myelination and remyelination of neurons, it is not a stretch to believe that it can be used to treat a patient suffering from traumatic nerve degeneration and/or a demyelinating disease of the CNS or PNS, such as MS, as is claimed in claim 12. The publications attached hereto include

in vivo results. Note, for example, the abstract of the *Molecular and Cellular Neuroscience* publication, which states that IL6RIL6 acts as an effective stimulator of the myelinating function of ES cell-derived oligodendrocyte precursors. Accordingly, as the present claims are now only directed toward myelination or remyelination, and as the evidence of record establishes that the chimera used in the present invention are useful for this purpose, it is not unreasonable to permit claims to the conditions specified in claims 11 and 12. Reconsideration and withdrawal of this part of the rejection are also respectfully urged.

Finally, the examiner states that the present claims are drawn to methods of using an IL6R/IL6 chimera but the present specification does not disclose which chimera is used. The examiner concedes that the specification states that the IL6R/IL6 chimera is a recombinant glycoprotein obtained by fusing the entire coding region of the naturally occurring human soluble interleukin-6 receptor δ-Val to the entire coding sequence of mature naturally occurring IL6. However, the examiner states that it is unclear which valine of the IL6R is being referred to or what is the structure of the human soluble IL6 receptor δ-Val. The examiner states that a search for human soluble interleukin-6 receptor δ-Val did not turn up any results. The examiner recognizes that the present

specification states that the chimera IL6R/IL6 is preferably produced in mammalian cells as described in WO 99/02552. However, the examiner states that this patent discloses several IL6R/IL6 chimeras and it is unclear which one of the chimeras disclosed therein is used in the claimed method. This part of the rejection is respectfully traversed.

With respect to IL6R δ-val/IL6, which the examiner states could not be found in the prior art, the examiner's attention is invited to WO 99/02552, referred to in the present specification at page 8, lines 3-6, which explicitly states that sIL6R δ-val/IL6 is the sequence having a tripeptide linker of E-F-M between the C-terminal Val-356 of sIL6R and the N-terminal Pro-29 of IL6, which protein has the sequence set forth in Figure 3. Thus, those of ordinary skill in the art reading the present specification know exactly what is the sequence of the IL6RIL6 chimera described at page 7, lines 19-28. Furthermore, there is no reason to believe that any of the other variations of this chimera that are disclosed as being equivalent in WO 99/02552 would not be considered to be equivalent to the sequence described in the present specification; they would all be expected to be equally effective in the present invention, whether or not they are explicitly claimed.

For all these reasons, reconsideration and withdrawal of this rejection is respectfully urged.

All the claims now present in the case clearly define over the references of record and fully comply with 35 USC 112. Reconsideration and allowance are therefore earnestly solicited.

Respectfully submitted,

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Original Article

Enhancement of Oligodendrocyte Differentiation from Murine Embryonic Stem Cells by an Activator of gp130 Signaling

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Key Words. *Embryonic stem cells · Glial differentiation · gp130 · IL-6R/IL-6 fusion protein · In vitro differentiation · Oligodendrocytes*

ABSTRACT

Embryonic stem (ES) cells derived from the inner cell mass of blastocyst-stage embryos are a potential large scale source of oligodendrocytes and of their progenitors for transplantation into the central nervous system for the repair of demyelinating lesions. We found previously that interleukin-6 (IL-6) fused to its soluble receptor (IL-6R), a potent activator of the gp130 receptor, induces myelin gene expression in Schwann cells of embryonic dorsal root ganglia. Like leukemia inhibitory factor, IL-6R/IL-6 inhibits the differentiation of murine ES cells into embryoid bodies. In the present study, we show that this recombinant cytokine may be efficiently used to stimulate the differentiation of oligodendrocytes if added to ES cell-derived neural precursors. IL-6R/IL-6 leads to an increase in early chondroitin sulfate proteoglycan positive and late O4 positive progenitors and to a stimulation of maturation into O1 and myelin basic protein expressing oligodendrocytes. Expression of the genes for transcription factor genes Olig-1 and Sox10, which appear early in the oligodendrocyte lineage, was stimulated by IL-6R/IL-6 addition. We conclude that this cytokine can significantly enhance the derivation of oligodendrocytes from ES cells. *Stem Cells 2004;22:344-354*

INTRODUCTION

Oligodendrocytes, which make the myelin sheaths in the central nervous system (CNS), evolve from multipotent neural stem cells (NSCs) through a series of developmental stages [1, 2]. The principal stages are: A) round pre-progenitors that express nestin as well as polysialylated neural cell adhesion molecule (PSA-NCAM) and which share with NSCs the property to grow as floating spheres when cultured in presence of growth factors [3, 4]; B) early bipolar progenitors or oligodendrocyte-type-2 astrocyte (O-2A) [5] staining with anti-ganglioside A2B5, and later forms becoming multipolar and positive for chondroitin sulfate proteoglycan (NG2); C) multipolar or arborizing late progenitors expressing O4 sulfatide glycolipids [6]; D) arborized premyelinating oligodendrocytes positive for galactocerebroside (GalC) and O1, and E) mature oligodendrocytes synthesizing the myelin membrane with its structural components such as myelin basic protein (MBP).

Embryonic stem (ES) cell lines derived from the inner cell mass of blastocysts are a potential large scale source of oligodendrocytes and of their progenitors, which have been used for transplantion into myelin deficient CNS [7-9]. Several culture conditions have been defined under which murine ES cells differentiating into embryoid bodies (EB) may be directed toward neural lineages, neurons, astrocytes, and oligodendrocytes. One approach is selection in serum-free defined medium

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in which neural precursor cells survive, proliferate in response to basic fibroblast growth factor (FGF-2), and differentiate when plated on adherent substrates after withdrawing the growth factor [10]. Some O4+ oligodendrocytes can then be derived provided tri-iodothyronine (T3) is added [10] in analogy to the effect of T3 on brain cell cultures [11]. Platelet derived growth factor (PDGF) promotes proliferation of brain glial precursors, in cooperation with epidermal growth factor (EGF) [12]. Similarly, on EB cells, combinations of FGF-2 with EGF and PDGF-AA provide more efficient growth of A2B5+ O-2A progenitors [8]. Yet another approach uses retinoic acid (RA) to induce neural precursors in mouse EB cultures [13, 14].

Brain NSCs can be further enriched by selecting nonadherent cells forming floating spheres in defined medium with FGF-2 or EGF and then by expanding them as multipotent neurospheres or as oligodendrocyte progenitor-enriched oligospheres [3, 15]. Similarly, oligospheres were obtained from RA-induced mouse EB cell cultures [14]. Floating neurospheres were also derived with FGF-2 from human ES cell lines and transplanted in vivo or plated in vitro on polycationic substrates yielding neurons, astrocytes, and oligodendrocytes [16, 17]. In these procedures, the growth factors used to obtain progenitors actually inhibit their differentiation [12]. A factor that stimulates oligodendrocyte differentiation from ES cells would therefore be of importance.

The cytokine ciliary neurotrophic factor (CNTF) has been reported to contribute to the proliferation of oligodendrocyte precursor cells from optic nerve [18]. However, the effect of CNTF on the differentiation of glial progenitors remains unclear as in several studies CNTF mainly induced astrocytes expressing glial fibrillary acidic protein (GFAP) with little effect on O4+ oligodendrocytes [5, 19-23], whereas in others it increased astrocytes and also survival and proportion of GalC+, O1+ and MBP+ cells in the cultures [24-26]. CNTF belongs to the interleukin-6 (IL-6) family of cytokines that signal via gp130 either as a heterodimer with the receptor for leukemia inhibitory factor ([LIFR] for CNTF, LIF, Oncostatin M[OSM]) or as a homodimer (for IL-6, IL-11) [27]. Differentiation of murine ES cells into EB is inhibited by LIF [28], CNTF [29], OSM [30] as well as by combinations or fusions of IL-6 with the extracellular portion of the soluble IL-6 receptor (sIL-6R) [31, 32]. Due to this inhibitory effect, the gp130 ligands were not investigated as differentiation agents for ES cells.

In previous studies we found that the IL-6R/IL-6 protein, a potent gp130 ligand in which IL-6 is fused to sIL-6R [33], is an efficient inducer of myelin gene expression in embryonic Schwann cells [34, 35] and an activator of myelin gene promoters [36]. Here we report that in cultures derived from murine ES cells, IL-6R/IL-6 strongly enhances the differentiation of early and late oligodendrocyte progenitors and their maturation into MBP+ cells.

Materials and Methods

ES Cell Cultures and Neurosphere Production

The murine ES cell line ROSA 11 [37] was maintained as before [38]. The cells were removed from the feeder layer with 0.05% trypsin and transferred without LIF to tissue culture plates in ES1 (Dulbecco's modified Eagle's medium [DMEM/F12] with 15% fetal calf serum [FCS], 1% glutamine, 0.1 mM β-mercaptoethanol, 2 μg/ml heparin) with 4 ng/ml of FGF-2. Media from (GIBCO/Invitrogen; Carlsbad, CA; http://www.invitrogen.com) were replenished daily. After 2 days the culture was treated with 0.2% dispase (GIBCO/Invitrogen) for 15 min at 37°C, and the clumps were reseeded into 9 cm tissue culture dishes in ES1 medium lacking FGF. After 4 days, clumps of differentiating EB cells were seen loosely bound to the dish by the intermediate of a few attached cells. The clumps, easily harvested using a needle, were transferred to new tissue culture dishes and cultured for another day in ES1 medium to facilitate attachment. Afterwards, selection for survival and growth of neural precursors [10] was achieved using EB-defined medium (DMEM/F12, 25 μg/ml insulin, 100 μg/ml transferrin, 60 μM putrescine, 30 nM sodium selenite, 2 μg/ml heparin, 20 nM progesterone) with 20 ng/ml of FGF-2. Medium was changed every 2 days. After 8-10 days, the cores of spherical aggregates containing neural precursors, as identified by surrounding radiating axons, were picked up with a needle. These spherical aggregates were then transferred into bacterial culture plates (Sterilin; Staffordshire, UK; http://www.bibby-sterilin.com) with the same EB-defined medium with 20 ng/ml FGF-2 and kept in suspension for at least 8 days. During the suspension culture, many cells detached from the aggregates and the latter acquired a regular shape typical of neurospheres [3]. Spheres expanding to more than 0.5 mm diameter were cut into two before reseeding in suspension for longer cultures. The composition of the floating neurospheres was examined after complete dissociation with 0.25% trypsin-EDTA by plating 25,000 cells on coverslips for immunostaining (as below).

Cell Differentiation Assay

Four neurospheres from the suspension cultures were deposited on each glass coverslip precoated with a solution of 20 μg/ml poly-D-lysine, 250 μg/ml fibronectin (PDL-FN), which were then placed into wells of 12-well plates in differentiation N2 medium (DMEM/F12 with 5 μg/ml insulin, 100 μg/ml transferrin, 16.1 μg/ml putrescine, 5.2 ng/ml

selenite, and 6.3 ng/ml progesterone—all added as 1% N2 supplement (from GIBCO). Laminin (2.5 μg/ml) and FGF-2, (5 ng/ml) were added for the first 4 days to facilitate attachment and then removed (laminin was omitted in some experiments without altering the results). Half of the wells were supplemented with IL-6R/IL-6 chimera, 100 or 200 ng/ml, produced in Chinese hamster ovary cells and purified as before [33]. The medium was replaced every 3 days, and in prolonged cultures 50 μg/ml ascorbic acid were added to the medium starting at day 21. At indicated times after plating the neurospheres, the cultures were fixed in 4% paraformaldehyde and kept in phosphate buffered saline/solution (PBS) at 4°C. After blocking with 5% normal goat serum (NGS), fixed cells were stained for early progenitors or oligodendroblasts with rabbit polyclonal anti-NG2 (chondroitin sulfate proteoglycan; Chemicon International; Temecula, CA; http://www.chemicon.com; 1:200) for 1 h at room temp (RT) and then Alexa Fluor 566-conjugated goat anti-rabbit antibody (Ab; Molecular Probes; Eugene, OR; http://www.probes.com; 1:250). Staining for late progenitors or pre-oligodendrocytes was with anti-sulfatide O4 mouse monoclonal (Mc) immunoglobulin M (IgM) antibodies (McAb 345 Chemicon; 1:75) for 1h at RT and fluorescein-conjugated goat anti-mouse IgM (Chemicon; 1:50). Immunostaining for GFAP, βIII-tubulin, nestin, and MBP was after permeabilization with 0.5% Triton-X100 and blocking with 10% NGS. Staining with mouse Mc anti-GFAP conjugated with the fluorescent Cy3 tag (Sigma; St Louis, MO; http://www.sigmaaldrich.com; 1:400) was for 1 h at RT. The mouse Mc immunoglobulin G (IgG) Tuj-1 anti-tubulin-βIII (Covance; Berkeley, CA; http://www.covance.com; 1:400) was used with goat anti-mouse IgG conjugated with Alexa Fluor 488 (Molecular Probes; 1:250). Staining for myelinating cells was with mouse Mc IgG anti-MBP (McAb 386; Chemicon; 1:400) and Cy3-conjugated affinity purified goat anti-mouse IgG, F(ab')2 fragment specific (Jackson ImmunoResearch Laboratories; West Grove, PA; http://www.jacksonimmuno.com; 1:400). Staining for neural precursors was with Mc IgG1 anti-nestin (Rat-401, Developmental Studies Hybridoma Bank at University of Iowa; Iowa City, IA; http://www.uiowa.edu/~dshbwww; 1:100) followed by the same Cy3-conjugated IgG as above.

Live cells were stained for O1. After blocking with 5% FCS, anti-O1 mouse IgM Mc antibodies (McAb 344, Chemicon; 1:75) and fluorescein-conjugated goat anti-mouse IgM (Chemicon; 1:50) were used for 1h at 37°C in humidified atmosphere, followed by fixation with 5% acetic acid in methanol. In all cases, the nuclear fluorescent dye DAPI (Sigma; 0.05 μg/ml) was added last. Coverslips were mounted in Mowiol (Calbiochem; La Jolla, CA; http://www.calbiochem.com), viewed in an Olympus IX-70 FLA microscope with a DVC-1310C digital camera (DVC; Austin, TX; http://www.dvcco.com) and images processed by Photoshop. Double-stained preparations are shown as overlayed images. A manual count program in the AlphaEase software (Alpha Innotech; San Leandro, CA; http://www.alphainnotech.com) was used to measure sizes and enumerate NG2, O4 and GFAP stained cells, as well as total cell nuclei visualized by DAPI.

Gene Expression Assays

Procedures for RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) for measuring levels of Sox10, GFAP, MBP and glyceraldehyde 3'-phosphodehydrogenase (G3PDH) gene transcripts were as described in detail previously, including the number of cycles and the primers used [36]. For the Olig-1 gene (accession NM_016968), the primers were: forward, 5'-TGCGCGCGAGAAGGCCGAAG and reverse, 5'-CCCAGCCAGCCCTCACTTG. Conditions for PCR amplification: 94°C, 2 minutes then 30 cycles at 94°C, 30 seconds; 56°C, 30 seconds; 72°C, 1 minute. The PCR buffer [36] was supplemented with 10% DMSO. After gel electrophoresis, the amplified DNA bands were photographed under UV-light, scanned, and their intensity was quantified using the AlphaEase spot density software. Band intensity was verified to be in the linear range by varying the amount of PCR reaction loaded on the gels.

Results

Addition Of IL-6R/IL-6 To EB Cell-Derived Neurospheres Enhances Oligodendrocyte Progenitor Differentiation

Like LIF and other members of the IL-6 cytokine family, IL-6R/IL-6 inhibited mouse EB formation (not shown). We, therefore, studied the effects of IL-6R/IL-6 when added to neurosphere cells derived from already preformed EBs. To produce neurospheres [16, 17], murine ROSA 11 ES cells, removed from the feeder layer, were induced to form EBs which were then subjected to selection for neural precursors [10] in serum-free medium supplemented with 20 ng/ml FGF-2. Under these conditions one observes the formation of spherical cell aggregates surrounded by outgrowing axons. The cores of these aggregates were dislodged and transferred to suspension culture, in which the floating spheres were maintained in the same selection medium containing FGF-2 for 8 or more days.

Dissociation of the floating spheres with trypsin and plating on glass coverslips confirmed that they are mainly composed of small round or elongated bipolar cells, 90% of which are positive for nestin, the intermediate filament protein found in neural precursors (Fig. 1A). Very few differentiating cells were seen at this stage (not shown), less than 1% staining for GFAP (astrocytes) or βIII-tubulin

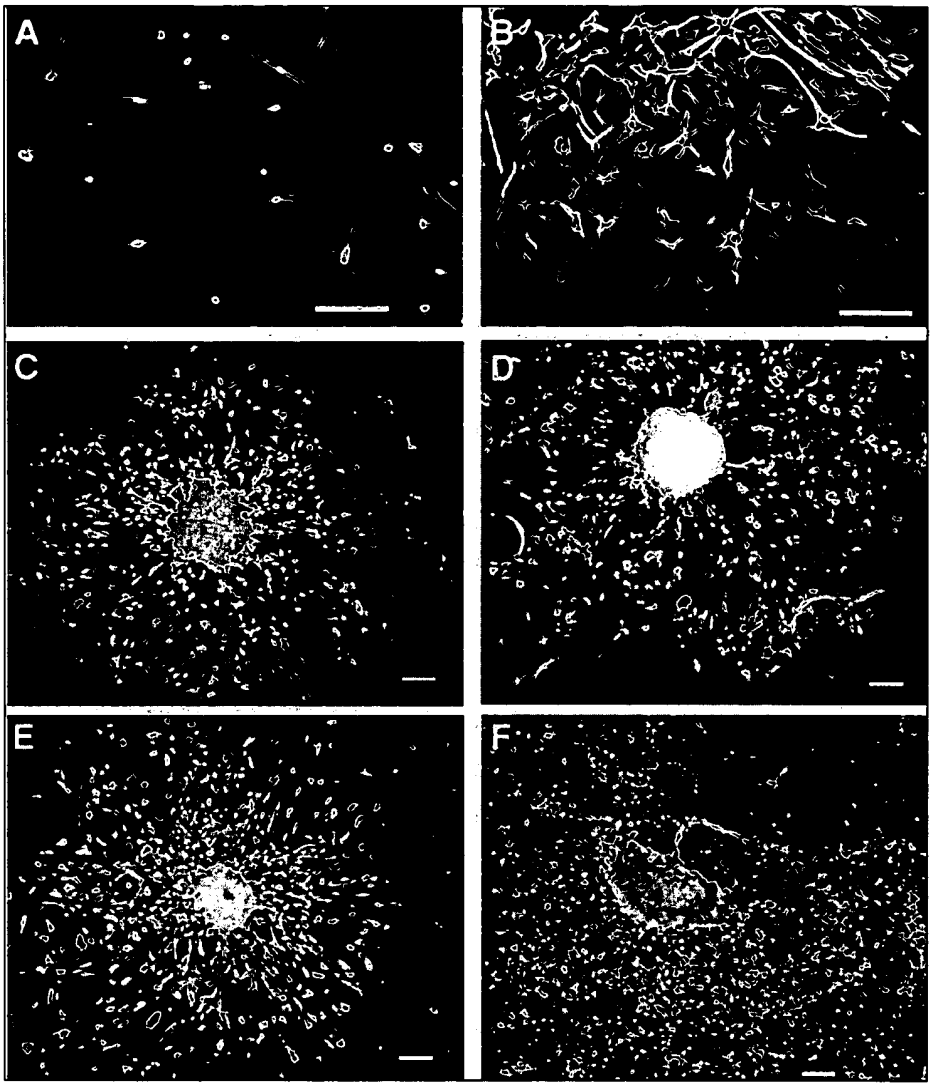

Figure 1. Neurosphere cells and effect of IL-6R/IL-6 on the outgrowth of glial cells. A) Mouse ES cell-derived floating neurospheres, after 19 days in suspension cultures, were dissociated with trypsin, plated, and stained for nestin (red) as marker of neural precursors and by DAPI (blue) to visualize nuclei. B) Outgrowth from neurospheres plated on PDL-FN adherent substratum double stained after the first 4 days for O4 (green) and GFAP (red). Note absence of O4⁺ cells. C) After 7 days of culture on PDL-FN (FGF removed at day 4), the outgrowth surrounding the neurospheres was double stained as in B and is seen to contain mainly GFAP⁺ astrocytes with few O4⁺ oligodendrocytes. D) Addition of 100 ng/ml IL-6R/IL-6 during the 7 days of culture on PDL-FN produces a large increase in O4⁺ oligodendrocyte progenitors (green). A series of enlarged microphotographs were used to quantify the results (Table 1). E) Culture after 15 days, under control conditions. F) After 15 days with IL-6R/IL-6, a large network of O4⁺ cells has formed. Size bars: 100 μm in all the panels.

(neurons), and none staining for the O4 sulfatide marking the progenitors differentiating into oligodendrocytes. We also examined cells staining for NG2, which was thought to be a specific marker of perinatal early oligodendrocyte progenitors but is now known to be also present in earlier neural multipotent precursors [39]. Up to 10% NG2⁺ cells were found, but all had a bipolar morphology resembling the other nestin-positive neural precursors present in the neurosphere and not oligodendrocyte progenitors (see below).

To investigate the effect of the gp130 activator IL-6R/IL-6 on differentiation, the floating spheres were placed on glass coverslips coated with PDL-FN, an adherent substratum that favors glial cell development [17], and the coverslips were incubated in defined N2 medium with or without IL-6R/IL-6 addition. To promote cell outgrowth, FGF-2 (5 ng/ml) and laminin (2.5 μg/ml) were added during the first 4 days, after which the cultures were continued without these additions. After the first 4 days, the outgrowth had formed a monolayer of GFAP⁺ cells but no O4⁺ cells were observed (Fig. 1B). On day 7 (i.e., 3 days after removal of FGF), O4⁺ oligodendrocyte progenitors became apparent in the control cultures (Fig. 1C), but their number and their size was much larger in the presence of IL-6R/IL-6 (Fig. 1D). In comparison, the GFAP⁺ astrocytes surrounding the neurosphere appeared similar in both conditions. Quantitative analysis of the stained cells versus the total cells labeled by DAPI showed that in the absence of IL-6R/IL-6, more than half the cells in the outgrowth were GFAP⁺ and about 2% were O4⁺ oligodendrocytes (Table 1). The data show that in the presence of IL-6R/IL-6 the proportion of GFAP⁺ cells was actually reduced whereas the percentage of O4⁺ cells became 6.4-fold higher than in the control conditions. IL-6R/IL-6 not only increased the proportion of O4⁺ progenitors, but accelerated their differentiation. Thus, at 15 days (Fig. 1F), the IL-6R/IL-6-treated cultures showed a marked expansion and branching of the O4⁺ oligodendrocyte progenitors, which formed a network over the astrocyte layer. Without IL-6R/IL-6, this differentiation was not apparent in the 15-day cultures (Fig. 1E).

In another experiment, the outgrowing cells were double stained for NG2 and O4 to compare the effect on early and late progenitors, respectively. To facilitate quantitative

Table 1. Effect of IL-6R/IL-6 on oligodendrocyte number and morphology

Experiment	Control conditions	With IL-6R/IL-6	*p* value
1. Percent O4$^+$ cells	2.2 ± 1	14.2 ± 5	0.004
Percent GFAP$^+$ cells	56.6 ± 18	30.6 ± 11	0.004
2. Percent O4$^+$ cells	0.8 ± 0.3	7.3 ± 1.7	0.002
Percent NG2$^+$ cells	4.2 ± 2.3	11.6 ± 2.8	0.007
3. Branch length (μm)	52 ± 19	211 ± 63	<0.0001

The mean percent of cells stained for O4, for GFAP, or for NG2 over total cell nuclei stained by DAPI is given with the standard deviation. For experiment 1 (7-day cultures) a total of 5,800 cells were counted in the outgrowth surrounding 14 attached neurospheres (half from cultures treated by IL-6R/IL-6) (Figs. 1C-1D). For experiment 2 (19-day cultures) a total of 6,800 cells were counted in the outgrowth of 10 neurospheres (half from cultures treated with IL-6R/IL-6), (Figs. 2A-2B). The lower percentage of O4 cells in this experiment is accounted for by the further proliferation of astrocytes (not shown). In experiment 3 (45-day cultures), the length of oligodendrocyte branches was measured on 25 cells (Fig. 4). Cell counting and measurements were done on microphotographs using the AlphaEase software. The *p* values were calculated by the Student's two-tailed *t*-test.

analysis in these 19-day cultures, the cells were observed at higher magnification (Figs. 2A-2B) which allowed clear visualization of the increase both in number and in size of the O4$^+$ progenitors produced by IL-6R/IL-6 treatment (Fig. 2B). The percentage of O4$^+$ cells rose from 0.8% to 7.3%, or 9.1-fold in response to the cytokine (Table 1). The percentage of NG2$^+$ cells was also increased in response to IL-6R/IL-6 but only by 2.7-fold (Table 1). The higher increment of O4$^+$ cells suggests that the main effect of IL-6R/IL-6 may be on the transition from NG2$^+$ early progenitors to more differentiated O4$^+$ cells. When examined at 7 days (Figs. 2C-2D), the increase in NG2$^+$ cells was similar (percentage increase of 2.5-fold). Although NG2 may also be present in multipotent neural progenitors [39], the NG2$^+$ cells in the outgrowth were mostly multipolar or branched (Figs. 2C-2D arrows) as typical for oligodendrocyte progenitors. Moreover, the size of the multipolar and branched NG2$^+$ cells was larger in the IL-6R/IL-6-treated cultures (Fig. 2D arrows). Hence, although less pronounced than for O4, the enhancement of NG2$^+$ early progenitors was reproducibly observed. On the other hand, when the cultures were stained for tubulin-βIII we did not observe significant changes in the density of the neuron axonal network in the outgrowth surrounding the neurospheres (Figs. 2E-2F). This makes it unlikely that the effect of IL-6R/IL-6 on oligodendrocyte differentiation would be a

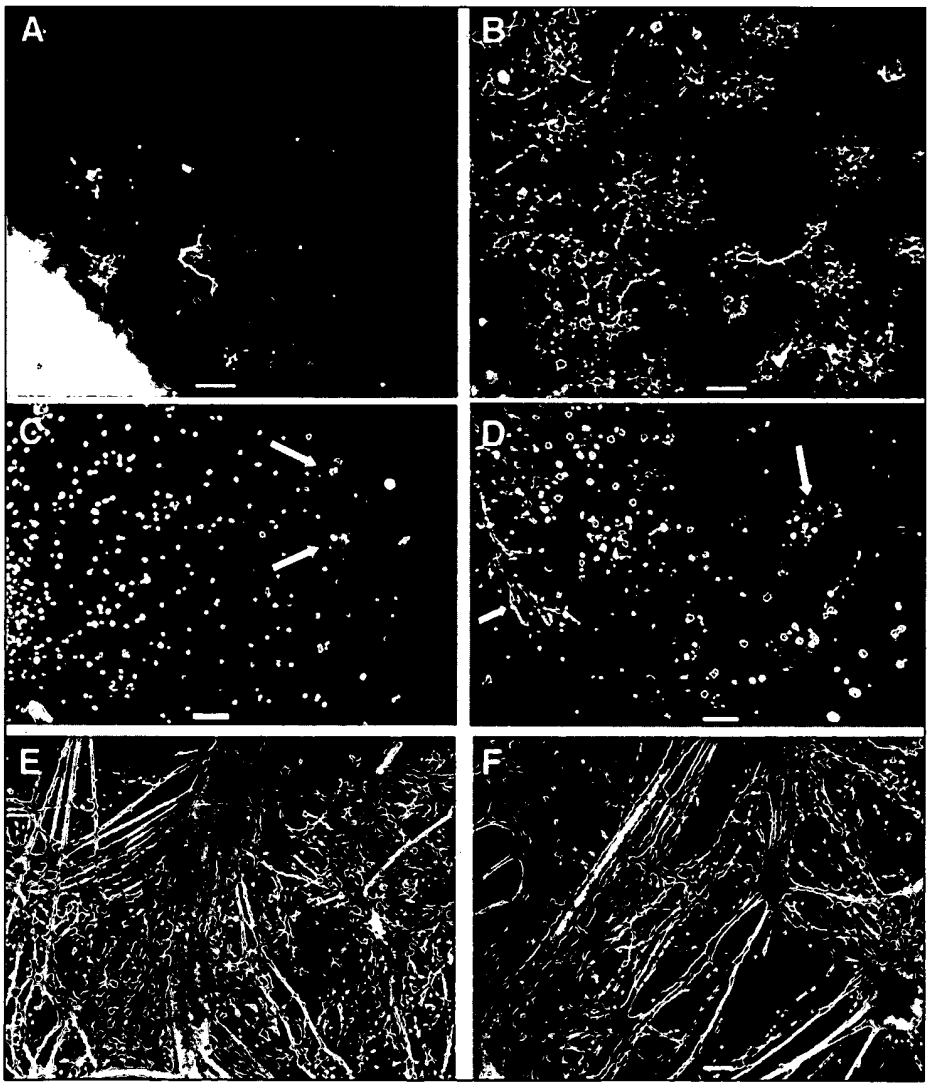

Figure 2. IL-6R/IL-6 increases differentiation of oligodendrocyte progenitors without affecting the axonal network. *A, B) Outgrowth from ES cell-derived neurospheres after 19 days on PDL-FN adherent substratum, fixed and double stained for NG2 (red, early oligodendrocyte progenitors) and for O4 (green, arborized late progenitors). The neurospheres are in the lower left corner. A) control culture, B) culture with 100 ng/ml IL-6R/IL-6, showing increase in stained cell number and size. A quantitative analysis appears in Table 1. C, D) similar cultures at 7 days, stained for NG2 (red) and by DAPI (blue) to visualize nuclei. C) control culture, D) culture with 100 ng/ml IL-6R/IL-6, showing increase in NG2$^+$ cell size and proportion (6.7% in control versus 16.9% with IL-6R/IL-6). E, F) Similar cultures for 21 days, fixed and stained to visualize the axonal network with anti-βIII-tubulin. E) control culture, F) culture with 200 ng/ml IL-6R/IL-6 (E, F same magnification). Size bars: 50 μm.*

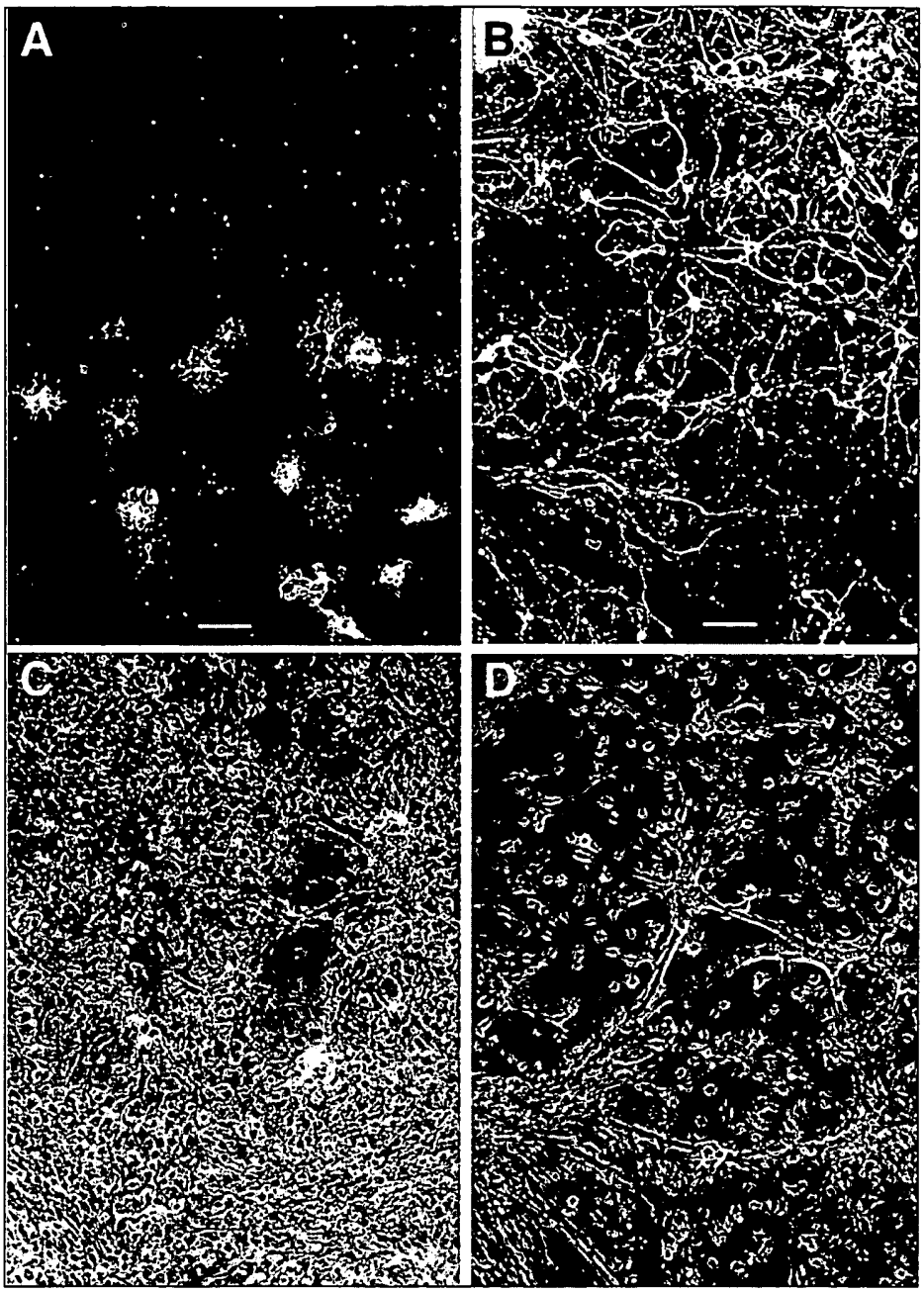

Figure 3. IL-6R/IL-6 enhances oligodendrocyte differentiation in long-term cultures. ES cell-derived neurospheres plated on PDL-FN and cultured for 6 weeks; outgrowing cells fixed and stained for O4. A) control culture, B) culture with IL-6R/IL-6, 200 ng/ml. The same fields are shown under light phase contrast. C) control culture, D) with IL-6R/IL-6. Under control conditions (A and C), a number of multipolar O4⁺ cells are seen within a monolayer of unstained cells. With IL-6R/IL-6 (B and D), a network of larger O4⁺ cells with long branches has developed, which represents the majority of the cells seen between thickened neuronal fibers. All panels at same magnification. Size bars: 100 μm.

secondary effect resulting from an increase in the axon network.

IL-6R/IL-6 Promotes Maturation of EB Cell Derived Oligodendrocytes

In long-term cultures (6 weeks), the control cultures developed small arborized O4⁺ oligodendrocyte progenitors (Fig. 3A), which were spread among the underlying layer of cells outgrowing from the neurosphere (Fig. 3C). In contrast, cultures treated with IL-6R/IL-6 formed a dense network of O4⁺ cells with considerably more arborization, which formed the majority of the cells in certain areas of the outgrowth and surrounded thickened nerve fibers (Figs. 3B, 3D). The O4⁺ oligodendrocytes in the IL-6R/IL-6 treated cultures reached much larger size than in the control cultures (Fig. 4, panels A, B, and E for untreated versus C, D, and F for IL-6R/IL-6-treated cultures). Measuring the length of the branches indicated a significant fourfold increase in the mean length as compared to control cultures (Table 1). Hence, while oligodendrocyte progenitors survived and increased in number in these long-term cultures under control conditions, the addition of IL-6R/IL-6 produced a notable expansion of the oligodendrocytes, both in number, in size, and in arborization.

Besides promoting oligodendrocyte differentiation, IL-6R/IL-6 also promoted their maturation. This is denoted first by the development of the cell processes into flattened myelin-like membrane sheaths that were visible in many cells from IL-6R/IL-6-treated cultures (Figs. 4C-4D, 4F). Such large membranes were not seen in untreated cultures (Figs. 4A-4B, 4E). Second, IL-6R/IL-6 enhanced the development of mature O1⁺ oligodendrocytes, whose size was considerably increased as compared to the control cultures (Fig. 5, panel A for control versus panels 5B-5C for IL-6R/IL-6-treated). In other experiments, we also found that the presence of IL-6R/IL-6 during the first 7 days was enough to produce the increase in O1⁺ cells at the end of the 6-week culture. Furthermore, the stimulating effect of IL-6R/IL-6 was similar in media supplemented with T3 and thyroxine (0.4 ng/ml each; not shown), indicating that the effect of IL-6R/IL-6 is in addition to that of these hormones.

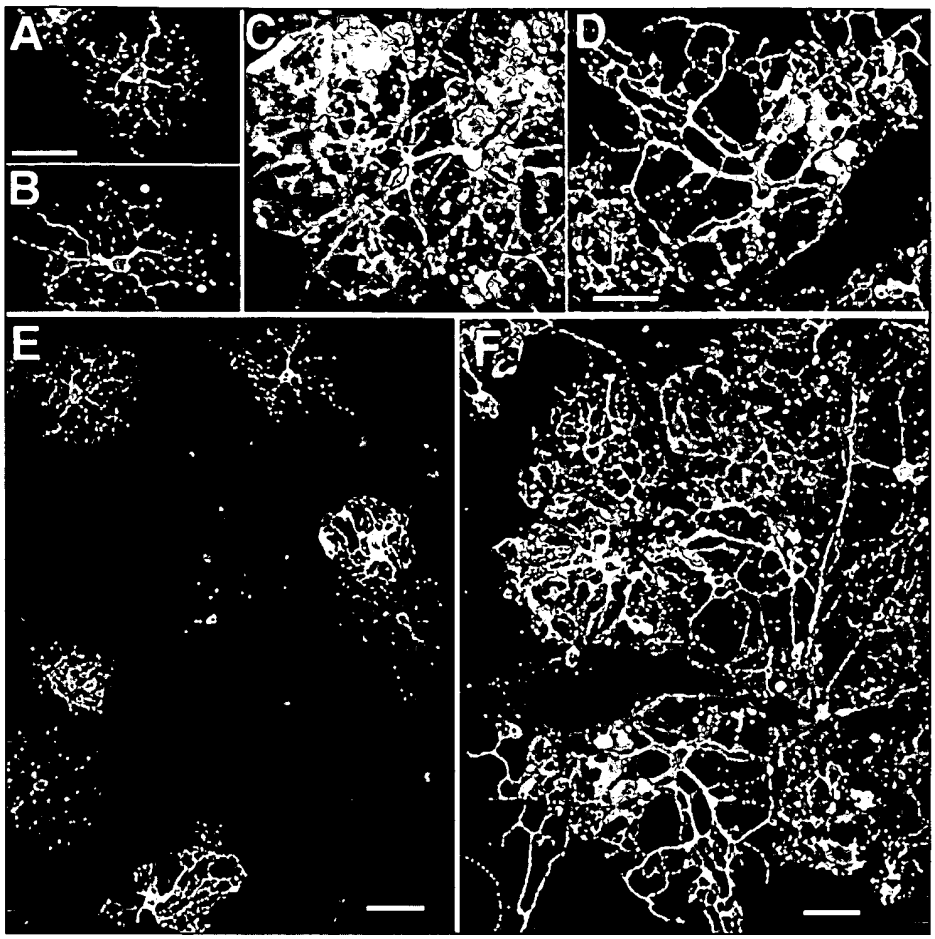

Figure 4. Comparative morphology of O4⁺ oligodendrocytes in ES cell-derived neurosphere cultures. A, B, E) Representative cells from control cultures; C, D, F) cells from IL-6R/IL-6-treated cultures (as in Fig. 3). With IL-6R/IL-6, the size and thickness of the oligodendrocyte branches increased markedly and myelin membranes were formed (see in panels C, D, and F). Quantitative results on branch length appear in Table 1. Panels A, B,and C, D are at the same 160× magnification. Size bars: 50 μm.

In line with the morphological development of myelin membranes, immunostaining for MBP was much higher in the oligodendrocyte network of IL-6R/IL-6-treated cultures at 6 weeks (Fig. 5E), than in the control cultures where only weakly labeled and small size cells were seen (Fig. 5D). Enhancement in MBP⁺ cells by IL-6R/IL-6 was already observed at 14 days (not shown). The gp130 activator, therefore, not only stimulated differentiation of ES-cell derived oligodendrocyte progenitors but also their maturation toward the myelinating phenotype.

IL-6R/IL-6 Enhances Oligodendrocyte Lineage-Specific Gene Expression

Olig-1 is a transcription factor of the bHLH group with a restricted expression seen in the oligodendrocyte lineage but not in astrocytes or other glial cells [40,41]. Olig-1 is expressed early and appears specifically required for the development and maturation of

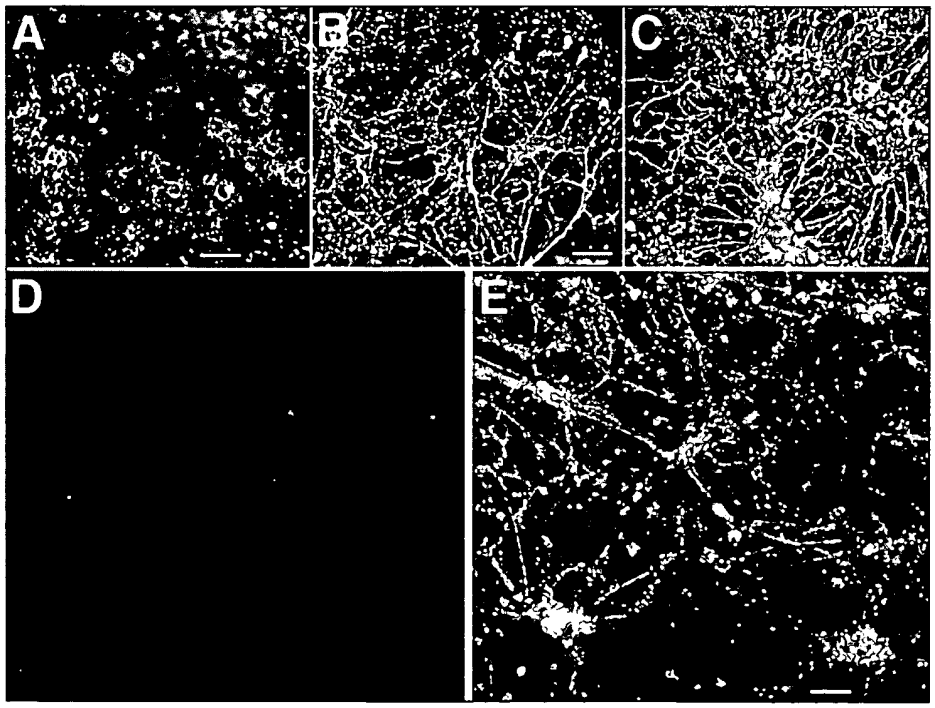

Figure 5. Increased oligodendrocyte maturation in response to IL-6R/IL-6. A-C) ES cell-derived neurospheres cultured for 6 weeks on PDL-FN and outgrowing cells stained live for O1. A) control culture; B, C) culture with IL-6R/IL-6, 200 ng/ml, which induces much larger branched O1⁺ oligodendrocytes. D, E) fixed cells stained for MBP. D) control culture shows weak MBP stain. E) culture with IL-6R/IL-6 200 ng/ml shows extensive accumulation of MBP. Panels A-C as well as D, E are at the same magnification. Size bars: 50 μm.

oligodendrocytes [42]. Sox10 is also expressed early in the oligodendrocyte lineage [41] and is a transcription factor acting on the promoters of myelin genes [36, 43]. The expression of these oligodendrocyte marker genes and of the astrocyte marker GFAP was examined by RT-PCR. We first analyzed RNA extracted from the spherical aggregates formed in the EB cultures after selection in serum-free medium with 20 ng/ml FGF-2 for 12 days. Little expression of Olig-1, Sox10, or GFAP RNA was detected in these spherical aggregates even when treated with IL-6R/IL-6 (Fig. 6, lanes 1-2). When RNA was extracted from outgrowing neurospheres cultured under the differentiation conditions on PDL-FN (4 days with 5 ng/ml FGF-2 and 2.5 μg/ml laminin and then four more days without these additions), expression of the three marker genes was observed (Fig. 6, lane 3). Addition of IL-6R/IL-6 for the last 4 days of this differentiation culture produced a marked increase in Olig-1 and Sox-10, whereas GFAP was unaffected (Fig. 6, lane 4). Furthermore, an induction of MBP RNA was observed in response to IL-6R/IL-6 (Fig. 6, lanes 5-6). Photometric scanning indicated increases of up to 20-fold for Olig-1 and 7.6-fold for Sox-10 in response to IL-6R/IL-6. In these short-term cultures, MBP was increased threefold. These gene expression profiles support the conclusion that the gp130 activator exerts enhancing effects on early phases of cell differentiation along the oligodendrocyte lineage (as denoted by Sox-10 and Olig-1 expression), as well as on the maturation toward myelinating MBP-expressing oligodendrocytes.

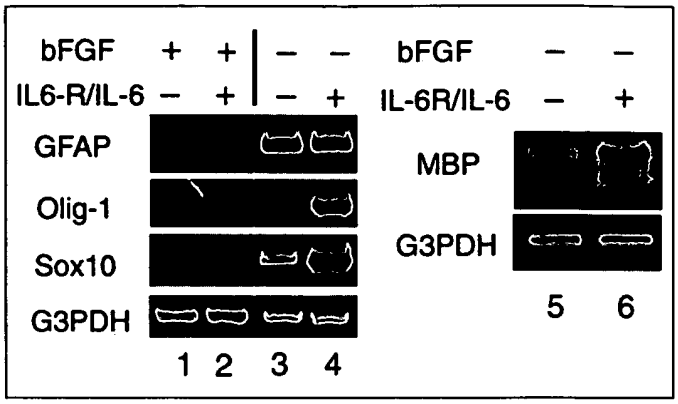

Figure 6. IL-6R/IL-6 enhances expression of oligodendrocyte lineage specific gene. Lanes 1, 2: gene expression in spherical aggregates formed in EB cultures after selection for 12 days in defined medium with 20 ng/ml FGF-2. Lanes 3-6: outgrowing neurospheres on PDL-FN for 8 days (FGF removed at day 4). Where indicated, IL-6R/IL-6 was added for the last 4 days before extracting RNA. For lane 2, IL-6R/IL-6 at 200 ng/ml and for lanes 4 and 6, IL-6R/IL-6 at 100 ng/ml. Expression levels measured by RT-PCR are shown for GFAP (astrocyte lineage), for Olig-1 and Sox10 (early oligodendrocyte progenitors), and for MBP (oligodendrocyte maturation), versus the housekeeping gene G3PDH as control for RNA loading.

Discussion

The IL-6R/IL-6 recombinant protein is a prototype of the IL-6 cytokine family. IL-6R/IL-6 has a high affinity for the gp130 receptor and can act on cells which do not respond to IL-6 itself [33, 44]. This results from the fact that the extracellular (soluble) portion of the gp 80 IL-6 binding receptor (sIL-6R) has the unusual property of being an agonist of IL-6 [45], so that in cells having only gp130 the IL-6/sIL-6R combination allows gp130 dimerization, thereby mediating signal transduction and activation of transcription factors of the STAT family [46].

This study demonstrates that a cytokine acting through the IL-6 family gp130 receptor may be effective in stimulating the differentiation of ES cell derivatives when added after the formation of EB. The differentiation-promoting activity of IL-6R/IL-6 was examined on EB-cell derived neurospheres consisting mainly of nestin positive neural precursor cells. Addition of IL-6R/IL-6 to neurospheres plated on an adherent substratum resulted in a marked increase in the total number and percentage of outgrowing O4$^+$ late oligodendrocyte progenitors. This effect of IL-6R/IL-6 was observed already after 7 days, and in longer term cultures IL-6R/IL-6 enhanced the differentiation of highly branched large O4$^+$ oligodendrocytes and their maturation into O1$^+$ and MBP$^+$ cells.

The steps leading to the development of the three main neural lineages from multipotent NSCs are still incompletely understood, in particular whether astrocytes and oligodendrocytes derive from a common glial SC or whether both neurons and oligodendrocytes derive from a common pathway distinct from that leading to astrocytes [42]. In the present experimental system, the proportion of GFAP$^+$ astrocytes among the cells outgrowing from the neurospheres was rather reduced by IL-6R/IL-6, and the density of the axons coming out from the neurospheres appeared unchanged (although we did not study the number of neuronal bodies inside the neurosphere). We focused on the action of IL-6R/IL-6 on differentiation of the oligodendrocyte lineage and examined whether IL-6R/IL-6 has an effect on early oligodendrocyte progenitors. Our experiments show that there is a moderate increase in NG2$^+$ early multipolar progenitors, although smaller than the increase in development of O4$^+$ late progenitors. Since no effect was seen on earlier bipotential O-2A A2B5$^+$ cells (not shown), the first target of IL-6R/IL-6 may be the NG2$^+$ committed progenitors. Further evidence for an early effect is seen in the expression of Sox10 and Olig-1 transcription factor genes, which are early markers of the oligodendrocyte lineage [40-42]. These genes started to be expressed when the neurospheres were plated on adherent substratum under conditions of outgrowth and differentiation following removal of FGF-2. However, supplementing these cultures with IL-6R/IL-6 caused within 4 days a marked increase in the expression of Sox10 and

Olig-1 genes, but not in the astrocyte GFAP marker gene. This suggests that IL-6R/IL-6 has some lineage-specific effects, such as the commitment or early differentiation of specific oligodendrocyte progenitors. This does not exclude that additional actions on cell growth and survival may be involved at this or later stages in the process of oligodendrocyte differentiation. In the control cultures, small arborized oligodendrocytes developed in the long-term cultures indicating that progenitors do survive. However, the mechanism(s) by which IL-6R/IL-6 caused the formation of much denser networks of larger long-branched oligodendrocytes and increased accumulation of MBP may be complex, and involve both enhancements of differentiation and of survival.

We previously showed that IL-6R/IL-6 induces myelin gene expression in Schwann cells from dorsal root ganglia of day-14 rat embryos [34]. Molecular effects of the gp130 ligand included a profound downregulation of transcription factor Pax3 [35], a step required for the onset of MBP gene expression [47]. Similarly, IL-6R/IL-6 activated the promoter transcriptional activities of the MBP and peripheral myelin Po genes in a transdifferentiating melanoma cell line, effects mediated in part by the downregulation of the Pax-3 repressor and by the increased expression of activators such as Sox10 [36]. The in vivo importance of gp130 signaling for Schwann cells has been shown by postnatal gp130 gene deletion which led, among others, to loss of Schwann cells and of peripheral myelin sheaths [48].

Effects of IL-6R/IL-6 could be observed also on neurospheres derived from post-natal mouse brain striatum, resulting in increased MBP mRNA (*Ben-Hur*, *Chebath* and *Revel*, unpublished observations). Increase of number of cells expressing a transgenic MBP promoter reporter gene in mouse brain cell cultures was recently reported in response to CNTF, LIF, OSM or the combination of IL-6 with sIL-6R [49]. In a previous study of the IL-6R/IL-6 protein using rat brain cortical cell cultures, a very marked increase in highly arborized oligodendrocytes was noted [50]. With IL-6R/IL-6 the number and length of MBP-stained branches was 2.3-fold larger than with CNTF, at their respective optimal doses [50]. In brain-derived cells, CNTF and LIF have been associated to astrocyte differentiation, with more variable effects on oligodendrocytes depending on the in vitro assays and type of cells examined [5, 19-26]. On the other hand, addition of CNTF to EGF-grown brain neurospheres was also reported to maintain NSC multipotency and reduce their transition to glial fate [23]. More recently, LIF was found to enhance formation of neurospheres, containing multipotent neural precursors, in sparse mouse ES cell cultures [51]. The differences in conditions and cells used in these studies make it difficult to compare the effects of CNTF or LIF to the action of IL-6R/IL-6 reported here, namely an enhancement of oligodendrocyte differentiation with no significant effect on astrocytes. This is the first study on ES cell-derived neurospheres showing that an IL-6 type cytokine stimulates the production of oligodendrocyte progenitors and their differentiation toward mature myelinating cells.

The potential of ES cell lines for expansion together with the availability of agents promoting their directed differentiation into various cell types holds much promise for the repair of injured tissues. Transplantations of murine ES cell-derived EBs and neurosphere cells have demonstrated remyelination, including examples of functional recovery [8, 9]. Recently, brain-derived neurospheres injected intravenously could induce recovery in a mouse model of multiple sclerosis [52]. Human ES cell lines have been used to prepare neuroglial cells transplantable into mouse brain [16, 17]. These strategies may lead to medical applications. IL-6R/IL-6 might have applications in the ex vivo preparation of myelinating cell transplants, or could be co-injected in vivo to stimulate differentiation of the myelinating cells in the CNS, as done previously to promote remyelination in rats subjected to sciatic nerve transections [35]. The improved derivation of oligodendrocytes from ES cells will also help in studying the molecular mechanisms and the control of their differentiation.

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References

1 Rogister B, Ben-Hur T, Dubois-Dalcq M. From neural stem cells to myelinating oligodendrocytes. Mol Cell Neurosci 1999;14:287-300.

2 Levine JM, Reynolds R, Fawcett JW. The oligodendrocyte precursor cell in health and disease. Trends Neurosci 2001;24:39-47.

3 Reynolds BA, Weiss S. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol 1996;175:1-13.

4 Ben-Hur T, Rogister B, Murray K et al. Growth and fate of PSA-NCAM$^+$ precursors of the postnatal brain. J Neurosci 1998;18:5777-5788.

5 Raff MC. Glial cell diversification in the rat optic nerve. Science 1989;243:1450-1455.

6 Schachner M, Kim SK, Zehnle R. Developmental expression in central and peripheral nervous system of oligodendrocyte

cell surface antigens (O antigens) recognized by monoclonal antibodies. Dev Biol 1981;83:328-338.

7 Brustle O, Spiro AC, Karram K et al. In vitro-generated neural precursors participate in mammalian brain development. Proc Natl Acad Sci USA 1997;94:14809-14814.

8 Brustle O, Jones KN, Learish RD et al. Embryonic stem cell-derived glial precursors: a source of myelinating transplants. Science 1999;285:754-756.

9 McDonald JW, Liu XZ, Qu Y et al. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. Nat Med 1999;5:1410-1412.

10 Okabe S, Forsberg-Nilsson K, Spiro AC et al. Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. Mech Dev 1996;59:89-102.

11 Barres BA, Lazar MA, Raff MC. A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. Development 1994;120:1097-1108.

12 Bogler O, Wren D, Barnett SC et al. Cooperation between two growth factors promotes extended self-renewal and inhibits differentiation of oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells. Proc Natl Acad Sci USA 1990;87:6368-6372.

13 Bain G, Kitchens D, Yao M et al. Embryonic stem cells express neuronal properties in vitro. Dev Biol 1995;168:342-357.

14 Liu S, Qu Y, Stewart TJ et al. Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. Proc Natl Acad Sci USA 2000;97:6126-6131.

15 Zhang SC, Lundberg C, Lipsitz D et al. Generation of oligodendroglial progenitors from neural stem cells. J Neurocytol 1998;27:475-489.

16 Zhang SC, Wernig M, Duncan ID et al. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 2001;19:1129-1133.

17 Reubinoff BE, Itsykson P, Turetsky T et al. Neural progenitors from human embryonic stem cells. Nat Biotechnol 2001;19:1134-1140.

18 Barres BA, Burne JF, Holtmann B et al. Ciliary neurotrophic factor enhances the rate of oligodendrocyte generation. Mol Cell Neurosci 1996;8:146-156.

19 Lillien LE, Sendtner M, Raff MC. Extracellular matrix-associated molecules collaborate with ciliary neurotrophic factor to induce type-2 astrocyte development. J Cell Biol 1990;111:635-644.

20 Gard AL, Williams WC 2nd, Burrell MR. Oligodendroblasts distinguished from O-2A glial progenitors by surface phenotype (O4+GalC-) and response to cytokines using signal transducer LIFR beta. Dev Biol 1995;167:596-608.

21 Bonni A, Sun Y, Nadal-Vicens M et al. Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. Science 1997;278:477-483.

22 Johe KK, Hazel TG, Muller T et al. Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev 1996;10:3129-3140.

23 Shimazaki T, Shingo T, Weiss S. The ciliary neurotrophic factor/leukemia inhibitory factor/gp130 receptor complex operates in the maintenance of mammalian forebrain neural stem cells. J Neurosci 2001;21:7642-7653.

24 Kahn MA, De Vellis J. Regulation of an oligodendrocyte progenitor cell line by the interleukin-6 family of cytokines. Glia 1994;12:87-98.

25 Mayer M, Bhakoo K, Noble M. Ciliary neurotrophic factor and leukemia inhibitory factor promote the generation, maturation and survival of oligodendrocytes in vitro. Development 1994;120:143-153.

26 Marmur R, Kessler JA, Zhu G et al. Differentiation of oligodendroglial progenitors derived from cortical multipotent cells requires extrinsic signals including activation of gp 130/LIFbeta receptors. J Neurosci 1998;18:9800-9811.

27 Taga T, Kishimoto T. Gp 130 and the interleukin-6 family of cytokines. Annu Rev Immunol 1997;15:797-819.

28 Williams RL, Hilton DJ, Pease S et al. Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 1988;336:684-687.

29 Conover JC, Ip NY, Poueymirou WT et al. Ciliary neurotrophic factor maintains the pluripotentiality of embryonic stem cells. Development 1993;119:559-565.

30 Rose TM, Weiford DM, Gunderson NL et al. Oncostatin M (OSM) inhibits the differentiation of pluripotent embryonic stem cells in vitro. Cytokine 1994;6:48-54.

31 Yoshida K, Chambers I, Nichols J et al. Maintenance of the pluripotential phenotype of embryonic stem cells through direct activation of gp130 signalling pathways. Mech Dev 1994;45:163-171.

32 Viswanathan S, Benatar T, Rose-John S et al. Ligand/receptor signaling threshold (LIST) model accounts for gp130-mediated embryonic stem cell self-renewal responses to LIF and HIL-6. STEM CELLS 2002;20:119-138.

33 Chebath J, Fischer D, Kumar A et al. Interleukin-6 receptor-interleukin-6 fusion proteins with enhanced interleukin-6 type pleiotropic activities. Eur Cytokine Netw 1997;8:359-365.

34 Haggiag S, Chebath J, Revel M. Induction of myelin gene expression in Schwann cell cultures by an interleukin-6 receptor-interleukin-6 chimera. FEBS LETT 1999;457:200-204.

35 Haggiag S, Zhang PL, Slutzky G et al. Stimulation of myelin gene expression in vitro and of sciatic nerve remyelination by interleukin-6 receptor-interleukin-6 chimera. J Neurosci Res 2001;64:564-574.

36 Slutsky SG, Kamaraju AK, Levy AM et al. Activation of myelin genes during transdifferentiation from melanoma to glial cell phenotype. J Biol Chem 2003;278:8960-8968.

37 Friedrich G, Soriano P. Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. Genes Dev 1991;5:1513-1523.

38 Li X, Chen Y, Scheele S et al. Fibroblast growth factor signaling and basement membrane assembly are connected during epithelial morphogenesis of the embryoid body. J Cell Biol 2001;153:811-822.

39 Belachew S, Chittajallu R, Aguirre AA et al. Postnatal NG2 proteoglycan-expressing progenitor cells are intrinsically multipotent and generate functional neurons. J Cell Biol 2003;161:169-186.

40 Zhou Q, Wang S, Anderson DJ. Identification of a novel family of oligodendrocyte lineage-specific basic helix-loop-helix transcription factors. Neuron 2000;25:331-343.

41 Wegner M. Expression of transcription factors during oligodendroglial development. Microsc Res Tech 2001;52:746-752.

42 Lu QR, Sun T, Zhu Z et al. Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. Cell 2002;109:75-86.

43 Stolt CC, Rehberg S, Ader M et al. Terminal differentiation of myelin-forming oligodendrocytes depends on the transcription factor Sox10. Genes Dev 2002;16:165-170.

44 Kollet O, Aviram R, Chebath J et al. The soluble interleukin-6 (IL-6) receptor/IL-6 fusion protein enhances in vitro maintenance and proliferation of human CD34(+)CD38(-/low) cells capable of repopulating severe combined immunodeficiency mice. Blood 1999;94:923-931.

45 Novick D, Shulman LM, Chen L et al. Enhancement of interleukin 6 cytostatic effect on human breast carcinoma cells by soluble IL-6 receptor from urine and reversion by monoclonal antibody. Cytokine 1992;4:6-11.

46 Murakami M, Hibi M, Nakagawa N et al. IL-6-induced homodimerization of gp 130 and associated activation of a tyrosine kinase. Science 1993;260:1808-1810.

47 Kioussi C, Gross MK, Gruss P. Pax3: a paired domain gene as a regulator in PNS myelination. Neuron 1995;15:553-562.

48 Betz UA, Bloch W, van den Broek M et al. Postnatally induced inactivation of gp 130 in mice results in neurological, cardiac, hematopoietic, immunological, hepatic, and pulmonary defects. J Exp Med 1998;188:1955-1965.

49 Stankoff B, Aigrot MS, Noel F et al. Ciliary neurotrophic factor (CNTF) enhances myelin formation: a novel role for CNTF and CNTF-related molecules. J Neurosci 2002;22:9221-9227.

50 Valerio A, Ferrario M, Dreano M et al. Soluble interleukin-6 (IL-6) receptor/IL-6 fusion protein enhances in vitro differentiation of purified rat oligodendroglial lineage cells. Mol Cell Neurosci 2002;21:602-615.

51 Tropepe V, Hitoshi S, Sirard C et al. Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. Neuron 2001;30:65-78.

52 Pluchino S, Quattrini A, Brambilla E et al. Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. Nature 2003;422:688-694.

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Increased myelinating capacity of embryonic stem cell derived oligodendrocyte precursors after treatment by interleukin-6/soluble interleukin-6 receptor fusion protein

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Neurosphere cells (NSc) derived from embryonic stem cells have characteristics of neural stem cells and can differentiate into oligodendrocyte precursors. Culture of NSc with IL6RIL6 chimera (soluble interleukin-6 receptor fused to interleukin-6) enhances their differentiation into oligodendrocytes with longer and more numerous branches and with peripheral accumulation of myelin basic protein (MBP) in myelin membranes indicating maturation. Gene expression profiling reveals that one of the proteins strongly induced by IL6RIL6 is a regulator of microtubule dynamics, stathmin-like 2 (SCG10/Stmn2), and gene silencing shows that Stmn2 plays an important role in the development of the mature oligodendrocyte morphology. IL6RIL6 acts as an effective stimulator of the myelinating function of ES cell-derived oligodendrocyte precursors, as observed upon transplantation of the IL6RIL6- pretreated cells into brain slices of MBP-deficient shiverer mice.

Introduction

Oligodendrocytes that produce myelin in the central nervous system (CNS) extend as many as 50 processes which wrap around axons to form myelin sheaths. In vitro and in vivo, the development of oligodendrocytes proceeds in steps from neural stem cells to bipolar progenitors, then to multipolar precursor cells having several main processes which subsequently arborize and form the multiple branches of the mature cells (Pfeiffer et al., 1993; Rogister et al., 1999). Oligodendrocytes precursors (OPCs) express the O4 sulfatide marker and successively galactocerebrosides (GalC, O1) and 2′,3′ cyclic nucleotide 3′ phosphodiesterase (CNP), and then develop into postmitotic maturating oligodendrocytes that synthesize myelin components such as proteolipid protein (PLP) and myelin basic protein (MBP) (Campagnoni et al., 1991; Warrington and Pfeiffer, 1992). In cultured oligodendrocytes undergoing maturation, the tip of the branches expand into broad sheets of myelin membranes, which resemble unwrapped myelin sheaths and can ensheath contacting axons (Knapp et al., 1987; Asou et al., 1995). During oligodendrocyte maturation, accumulation of MBP in the peripheral branches and myelin membrane sheets results from the transport of MBP mRNA along microtubules, a process mediated by kinesin (Trapp et al., 1987; Verity and Campagnoni, 1988; Carson et al., 1997). Elongation of branches and formation of myelin membranes involve different cytoskeleton rearrangements: from the perikaryon, microtubules extend in the major processes, branches and veins, while not penetrating into the flattened membrane sheets where the cytoskeletal infrastructure is a meshwork of microfilaments (Carson et al., 1997; Song et al., 2001). Understanding the process of oligodendrocyte maturation and identifying factors that stimulate terminal differentiation could help explain how these cells generate and maintain the large amount of membranes required for CNS myelination and how this process may be enhanced.

Another long-term goal of culturing neural stem cells and oligodendrocyte progenitors is to obtain populations of cells for transplantation with the aim of remyelinating CNS regions with lesions from traumas or various pathologies (McKay, 1997; Cao et al., 2002). Eventually, cell replacement may allow to achieve regional or even global repair of myelin as indicated by experiments in MBP-deficient shiverer mice (Lachapelle et al., 1983; Gumpel et al., 1989; Yandava et al., 1999). Embryonic stem (ES) cell lines being amenable to mass culture and differentiation into specific cell lineages in vitro are a potential large scale source of oligodendrocytes for brain and spinal cord transplantation, which has been studied in experimental models using mouse (Brustle et al., 1999; McDonald et al., 1999; Liu et al., 2000; Glaser et al., 2005) and human ES cells (Reubinoff et al., 2001; Zhang et al., 2001; Nistor et al., 2005). For successful

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transplantation, the cells need the capacity to migrate into the CNS and migration of OPCs depends on their differentiation stage: more differentiated oligodendrocytes migrate minimally but conversely the cells that migrate more are less likely to differentiate into mature myelinating cells (Warrington et al., 1993; Foster et al., 1995; Yandava et al., 1999). Although the CNS seems to provide endogenous regional signals that will cause incoming OPCs to differentiate, it may be of interest to find factors that can be administered exogenously to potentiate the maturation of the cells and their capacity to myelinate nerves.

Growth factors such as Epidermal growth factor (EGF), Fibroblast growth factor bFGF (bFGF) and Platelet-derived growth Factor (PDGF-AA) stimulate proliferation of neural stem cells and very early glial progenitors, allowing their isolation as floating neurospheres, but these factors do not support or even inhibit advanced differentiation (Bogler et al., 1990; Gard and Pfeiffer, 1993; Vescovi et al., 1993; Reynolds and Weiss, 1996). Factors that promote differentiation of oligodendrocyte precursors include (1) thyroid hormone, triodo-thyronine or T3 (Barres et al., 1994a), (2) neurotrophin NT-3 (Barres et al., 1994b; Heinrich et al., 1999) which particularly when transfected in the cells increases MBP and myelination (Rubio et al., 2004), (3) cytokines of the Interleukin-6 (IL-6) family. Among the latter, Ciliary neurotrophic factor CNTF was shown in some culture system to stimulate differentiation into MBP- and O1-positive cells (Mayer et al., 1994; Marmur et al., 1998) or increase the length of $O4^+$ branches (MacDonald et al., 2002), while in other systems, CNTF acted only on survival and growth of early progenitors (Barres et al., 1993, 1996) and increased astrocytosis (Lillien et al., 1990; Gard et al., 1995). We study a fusion protein linking IL-6 to its soluble receptor (IL6RIL6) thereby forming a high-affinity activator of the gp130 receptor (Chebath et al., 1997; Fischer et al., 1997; Kollet et al., 1999), the common signaling receptor for all members of the interleukin-6 (IL-6) cytokine family and one of the components of the CNTF receptor system (Taga and Kishimoto, 1997). In brain primary cultures, IL6RIL6 has been more potent than CNTF to stimulate differentiation of highly arborized oligodendrocytes and increase the number and length of their branches (Valerio et al., 2002). We previously reported that IL6RIL6 addition to ES cell-derived whole neurospheres stimulates the development of oligodendrocytes growing out from the neurospheres (Zhang et al., 2004). The present study shows that neurosphere cells (NSc) can be dissociated, propagated with growth factors and then respond to stimulation by IL6RIL6 to undergo maturation and acquire MBP-containing peripheral myelin membranes. In transplantation assays, IL6RIL6 greatly improves the myelinating capacity of ES-derived NSc for CNS neurons in brain tissue from shiverer mice.

Results

Effect of IL6RIL6 on differentiation of ES cell-derived neurosphere cells

ES cell-derived dissociated neurosphere cells (ES-NSc), which have the morphology of small nestin-positive neural progenitors (Zhang et al., 2004), developed into $O4^+$ oligodendrocyte precursors when cultured on polyornithine in defined medium with EGF and bFGF for 2 days and then 9 days without growth factors (Fig. 1A). When IL6RIL6 was added throughout the culture, there was a marked increase in the size of the $O4^+$ cells which is due to a much greater length and complexity of branches and the formation of membrane sheets (Fig. 1B). On the average, the diameter of the $O4^+$ cells increased from 57 ± 19 μm to 172 ± 32 μm in the IL6RIL6-treated cultures. The percentage of $O4^+$-positive cells over total cells (DAPI) was also increased from 20.6% ± 1 to 38.2% ± 3.

Addition of IL6RIL6 toward the end of the culture produced similar effects on oligodendrocyte development. ES-NSc were cultured 3 days with EGF and bFGF, then 3 days without growth factors, following what the culture was continued 6 days without or with IL6RIL6 (Figs. 1C, D). The $O4^+$-positive cells in the IL6RIL6-treated culture were much more developed and formed parallel arrays of branches sometimes several hundred microns long (panel D). Examining other neural lineages showed that the ratio of $GFAP^+$ astrocytes to $O4^+$ cells was around 0.5–0.6 in the control cultures (panel C) and was similar with IL6RIL6 although the $GFAP^+$ cells were now clustered at the periphery of the large oligodendrocytes (panel D). Neurons stained for tubulin-βIII developed interspersed with small oligodendrocytes in the control conditions (Fig. 1E) whereas with IL6RIL6 the oligodendrocytes almost covered the neuronal network (panel F). Contacts between well-arborized oligodendrocytes and axons from several neurons could be seen at higher magnification (panel G). In the experiments shown in panels E–G, the neurosphere cells were cultured with EGF + PDGF following the EGF + bFGF step, then without growth factors while IL6RIL6 was added only for the last 3 days of these 12-day cultures. Even shorter treatments of 1–2 days with IL6RIL6 given at the end of the culture already produced marked increase in the number of $O4^+$ cell branches (not shown). Thus, the differences in size and morphology of oligodendrocytes resulting from IL6RIL6 treatment were consistently observed in cultures with various schedules and of different durations, from a few days and up to 6 weeks.

Time-course experiments showed that the ES-NSc start to express myelin gene transcripts in the 3–6 days after removal of growth factors, first CNP then MBP (see Supplementary Fig. 1, on line). Hence, when IL6RIL6 addition is delayed, the marked effect on oligodendrocyte development denotes action on a relatively late stage of differentiation after the onset of MBP gene transcription. Evidence for this late effect was substantiated by investigating the intracellular distribution of MBP.

IL6RIL6 promotes oligodendrocyte maturation with MBP in peripheral branches and myelin membranes

When ES-Nsc were differentiated for 12 days and double stained for MBP and O4, a marked change in the distribution of MBP was observed in response to IL6RIL6. Without IL6RIL6 treatment (Fig. 2A), MBP was restricted to the perikaryon and cell body. In cultures exposed for the last 3 days to IL6RIL6, the MBP label extended along the branches and in thickened processes (Fig. 2B). Furthermore, when IL6RIL6 was added for longer periods, most of the MBP was located in large sheets that expand from the $O4^+$ branches (Fig. 2D). Such sheets did not form in the absence of IL6RIL6 (Fig. 2C). A higher magnification of the IL6RIL6-treated

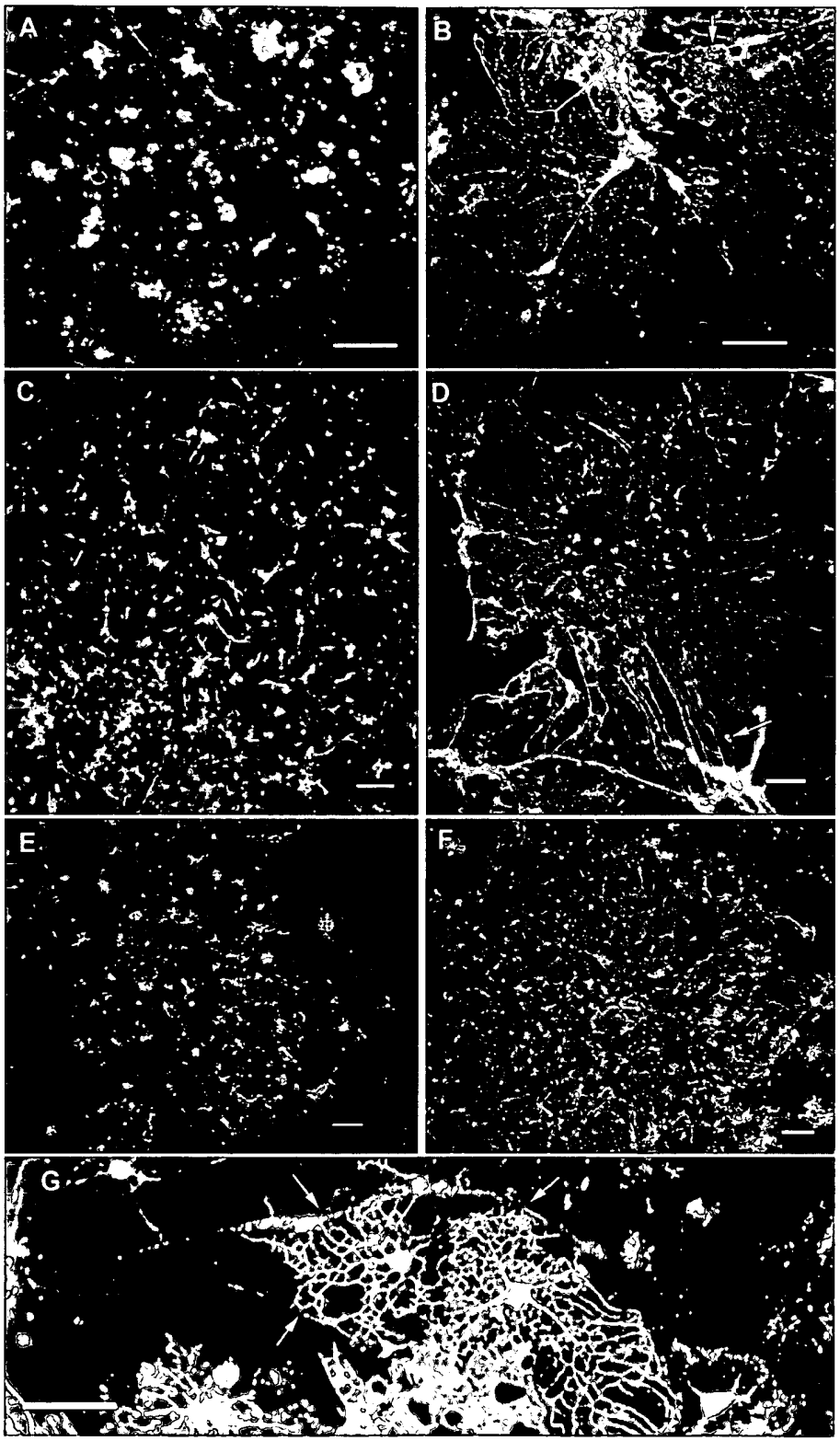

Fig. 1. Increased development of oligodendrocytes in response to the gp130 activator IL6RIL6. (A, B) ES cell derived dissociated neurosphere cells (ES-NSc) plated for 2 days with EGF and bFGF then 9 days without growth factors (A) and same with IL6RIL6 throughout (B). Note increase with IL6RIL6 in number and size of $O4^+$ (green) branches and membrane sheets (arrow in B). Nuclei stained with DAPI (blue). (C, D) ES-NSc for 3 days with EGF and bFGF, then without for 9 days (C) and same with IL6RIL6 added only for the last 6 days (D). IL6RIL6 increases the size of $O4^+$ processes, forming parallel bundles of fibers (arrow in D), with $GFAP^+$ cells (orange) at the outside. (E, F) ES-NSc for 3 days with EGF and bFGF, then 3 days with EGF and PDGF, then 6 days without (E) and same with IL6RIL6 added for the last 3 days (F). IL6RIL6 induces a dense array of $O4^+$ branches covering the neuronal network (tubulin-βIII^+, orange-red). (G) At higher magnification, IL6RIL6-treated oligodendrocytes with extensive $O4^+$ arborization contact axons (red, arrows). Size bars: A–F, 100 μm; G, 50 μm.

cells shows the flattened membrane sheets forming in between the branches and veins and the accumulation of MBP (Figs. 2E–G). These areas resemble myelin membranes of mature oligodendrocytes and probably correspond to unfurled myelin sheaths, as observed with brain-derived oligodendrocyte cultures (Carson et al., 1997). A more detailed time course experiment indicated that IL6RIL6 treatments of 1–3 days are sufficient to cause extension of branches, whereas the formation of the broad MBP-containing sheets is only observed with longer treatments (not shown). Thus, IL6RIL6 appears to induce a progressive maturation event.

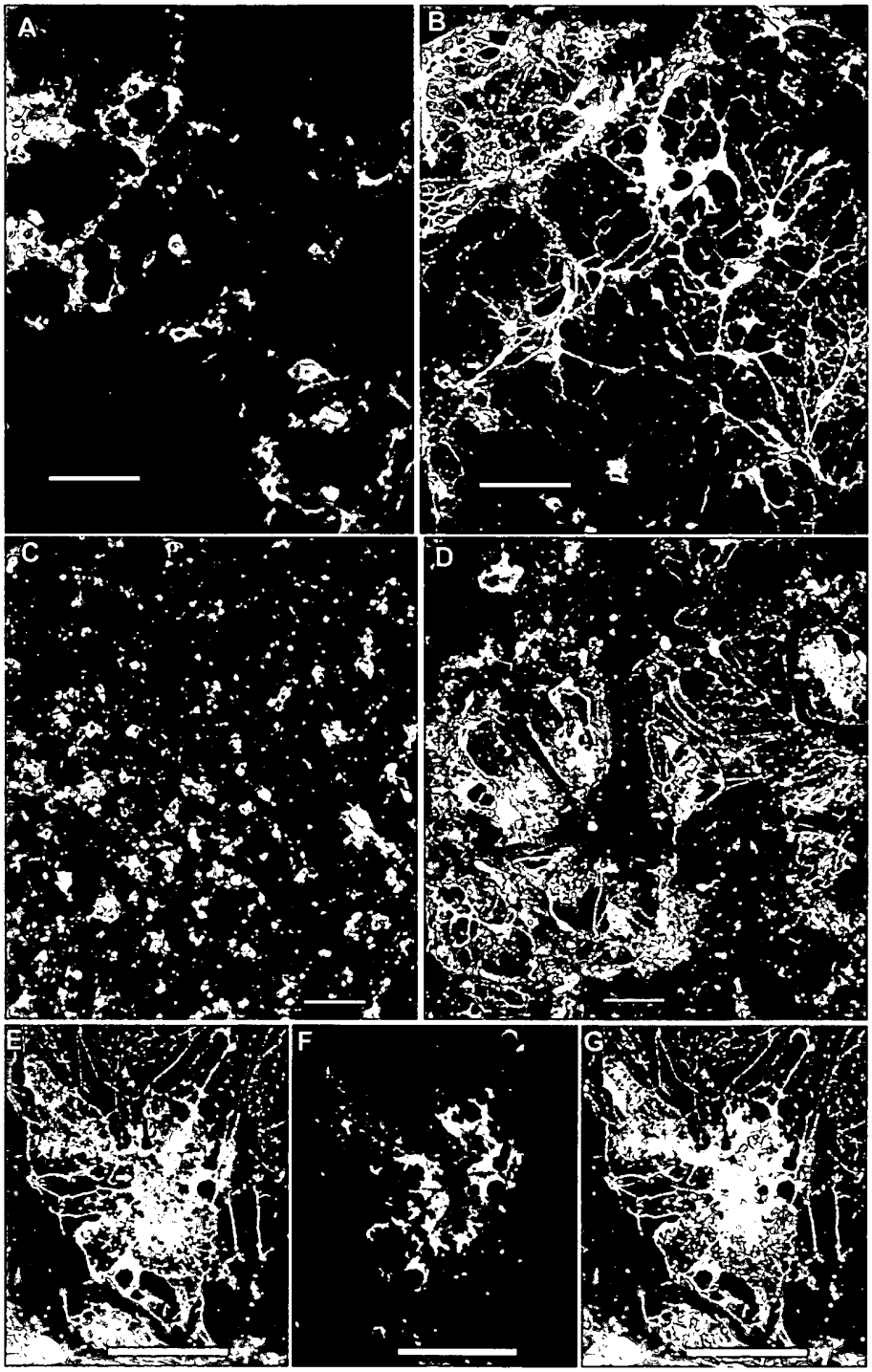

Fig. 2. Distribution of MBP and formation of myelin membranes in response to IL6RIL6. Immunostaining for MBP (red) appears yellow when overlapping O4 (green). (A, B) ES-NSc cultured 3 days with EGF and bFGF, 3 days with PDGF, then without growth factors for 6 days (A) or same with IL6RIL6 added for the last 3 days (B). Note increased distribution of MBP along the branches in response to IL6RIL6. (C–G) ES-NSc (after 2 passages) cultured 2 days with EGF and bFGF and 9 days without (C) or with IL6RIL6 throughout (D). With prolonged IL6RIL6 treatment, numerous broad sheets containing MBP appear (arrows), which have the typical appearance of myelin membranes extending from the branches. Higher magnification shows O4 stained branches and sheet (E, arrow), with MBP accumulating in the sheet between veins (F is MBP stain; G is overlap of MBP and O4). Size bars: 100 μm in all.

The mechanism by which MBP localizes in the distal cell branches and myelin membranes of oligodendrocytes is known to be dependent on the transport of MBP mRNA transcripts by kinesin-driven granules moving along microtubules (Carson et al., 1997). Many cytoskeletal rearrangements are essential in the development of oligodendrocyte branches and myelin membranes (Song et al., 2001). We therefore searched for gene products that are induced by IL6RIL6 treatments and could impact on cytoskeleton assembly and function.

IL6RIL6 induces a regulator of microtubule dynamics

Gene expression profiling by DNA microarrays (Affymetrix, see Supplementary Results) was carried on ES-NSc that were cultured 3 days with EGF and bFGF, 3 days with EGF and PDGF-AA and then without growth factors for 6 days. In half the cultures, IL6RIL6 was added for the last 3 days. Among the genes that were induced by IL6RIL6 to high expression levels, we found the cytoskeleton-related gene SCG10 (supercervical ganglia protein 10 or stathmin-like 2, Stmn2; NCBI: BM115022, BM946869, NM_025285) which was induced by 10-fold (see Supplementary Table 1, on line). SCG10 has been described in neurons as a regulator of microtubule dynamics and inhibitor of tubulin polymerization (Gavet et al., 1998; Grenningloh et al., 2004). Immunoblots of total cell extracts showed that the SCG10 protein was induced by IL6RIL6 in the ES-NSc cultures, with a maximum when IL6RIL6 treatment was for the last 3–6 days (Fig. 3A). In the cultures without IL6RIL6, double staining with antibodies to SCG10 and to tubulin-βIII showed that the SCG10 protein was present mainly in neurons (Fig. 3B, arrows). When IL6RIL6 was added for the last 3 days of culture, there was a marked accumulation of SCG10 in multi-branched cells (Fig. 3C). These cells, which were clearly distinct from the tubulin-βIII labeled neurons, accounted for most of the induction of SCG10 as compared to the cultures without IL6RIL6.

Double staining for O4 and SCG10 demonstrated that SCG10 was indeed induced by IL6RIL6 in the differentiating oligodendrocytes (Figs. 3D–F). In ES-NSc treated for only 2 days with IL6RIL6, the multiple branches labeled by O4 antibodies (green) were positive also for SCG10 (red) and the two labels clearly overlapped (panel F, yellow). Interestingly, SCG10 (but not O4) appeared to accumulate at discrete sites often located near turns and bifurcation of the branches (panel E and F, line and arrows). Many more such clusters of SCG10 accumulation were apparent along the branches of IL6RIL6-treated cells (panel H), than in the untreated cells where label was mainly in the cell body (panel G). The timing of SCG10 induction by IL6RIL6, along with its discrete localization along the branches, would be in line with a role for this regulator of microtubule dynamics in the effect of IL6RIL6 on the development of the oligodendrocyte multiple branches.

Inhibition of SCG10/Stmn2 gene expression by transfecting siRNA into cultures of ES-NSc profoundly affected the morphology of the oligodendrocyte branches (Figs. 3I and J). In the control culture, mock-transfected and treated 5 days with IL6RIL6, the $O4^+$ cells (brown) exhibited the usual development of a dense network of long branches and of membrane sheets (panel I, arrow). This network was absent from cultures transfected by SCG10 siRNA (panel J) and instead some of the $O4^+$ cells developed abnormally long and straight processes with little branching (arrow). The abnormal morphology of the $O4^+$ cells was observed 48 h after transfection with SCG10 siRNA as compared to transfection with control siRNA (Figs. 4A–D). It was seen in the absence of IL6RIL6 (panels A, B) and more clearly when the cells were treated for the last 24 h with IL6RIL6 (panels C, D). The formation of multi-branched oligodendrocytes and the marked increase in small sub-branches (arborization) following IL6RIL6 treatment was inhibited by SCG10 siRNA, so that cells had only a few processes that were longer and thicker but largely devoid of sub-branching or veins. Under the conditions of Figs. 4C, D, a statistical analysis of the O4-positive cells showed that 85 ± 11% of these had the normal multi-branched phenotype when control siRNA was applied, versus 13 ± 8% when SCG10 siRNA was applied ($N = 60$ cells each; $P < 0.0001$).

When the cells were stained for both O4 and SCG10, the decrease in SCG10 could be visualized in the cells lacking sub-branching (Fig. 4, panel C versus D). Immunoblots indicated that the overall reduction in SCG10 protein by the specific siRNA was about 60%, and using fluorescent si*GLO* Risc-free as a tracer we estimated the efficiency of siRNA uptake in these experiments to be also 60% (not shown). Examining cells exhibiting uptake of the tracer mixed with SCG10 siRNA (at 2 days post-transfection) showed that these cells lacked branching, whereas, in the same field, neighboring cells that did not take up siRNA had multi-branched morphology (Fig. 4E). At 4 days after transfection (the 3 last days with IL6RIL6), the reduction of SCG10 was still visible when comparing cells with abnormal morphology (Figs. 4F, G, arrowhead) to multi-branched cells in the same field (arrow). At this time point, we determined the percentage of total cells (as visualized with DAPI) which had an $O4^+$ multi-branched morphology to be 17 ± 2.7% with control siRNA versus 5.5 ± 2.7% with SCG10 siRNA ($N = 600$ cells each; $P = 0.0075$). Conversely, the percentage of cells having abnormal morphology and lack of sub-branching was 8.5 ± 0.6% with SCG10 siRNA versus 1 ± 0.4% with control siRNA ($P < 0.0001$).

The abnormal development of oligodendrocytes in cultures transfected by SCG10 siRNA was further evidenced in other experiments where the cells were double stained for O4 and MBP after 5 days of treatment with IL6RIL6 (Figs. 4H, I). With control siRNA (panel H), MBP was distributed along the extensive branching network and accumulated in membrane sheets, as seen above. With SCG10 siRNA, there was a clear reduction of the network of small $O4^+$ sub-branches and MBP staining was seen only around the cell body (panel I, arrowhead) despite the formation of $O4^+$ membrane sheets (arrows). IL6RIL6-inducible SCG10/Stmn2 appears therefore involved also in the peripheral accumulation of MBP, but does not appear required for membrane sheet formation.

Increased myelination capacity of ES cell derived neurosphere cells treated by IL6RIL6

The myelinating capacity of oligodendrocyte precursors obtained under various culture conditions was compared by transplantation into brain slices from MBP deficient shiverer mice. Since homozygous shiverer mice (*shi/shi*) have an extensive deletion of five exons of the MBP gene (Roach et al., 1985) and no MBP immunoreactivity in CNS, immunostaining for MBP may be used as a marker for remyelination by donor oligodendrocytes or precursor cells (Lachapelle et al., 1983; Yandava et al., 1999). Organotypic CNS slice cultures have been used as host for the

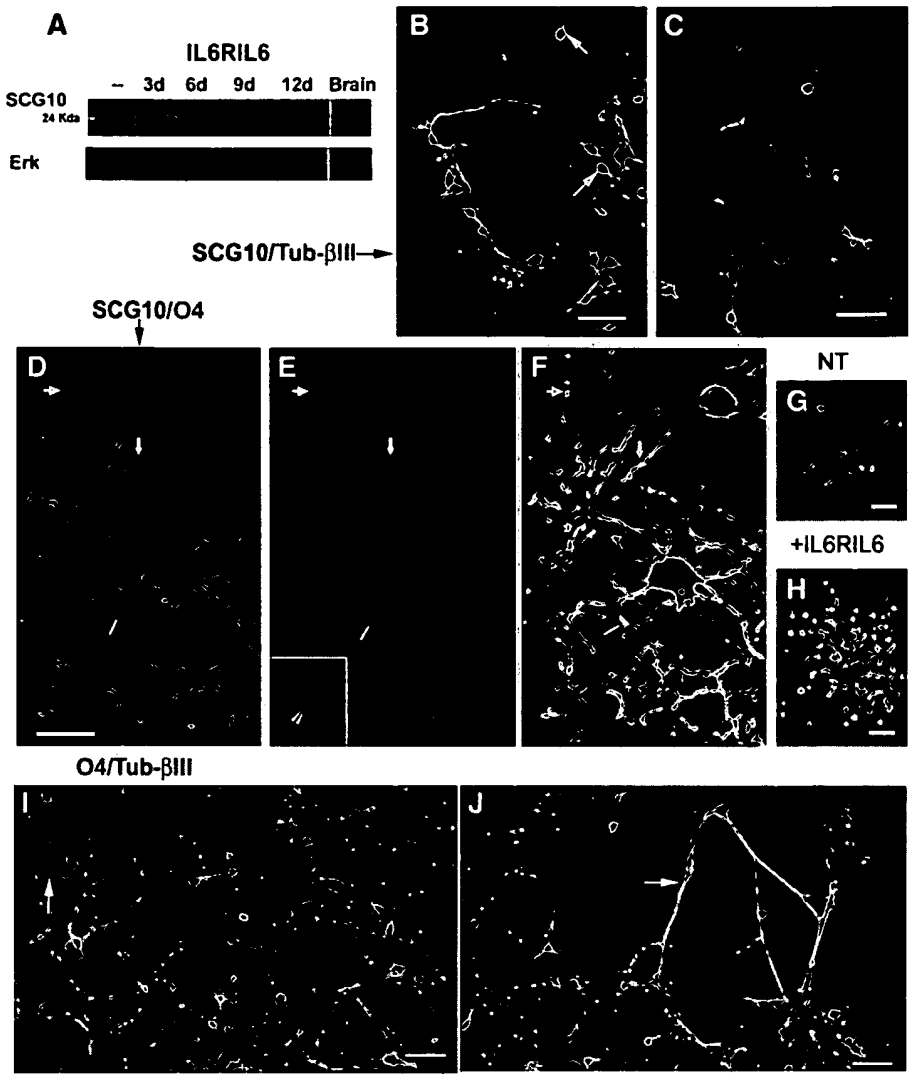

Fig. 3. SCG10 (Stathmin-like 2) induction by IL6RIL6. (A) Immunoblot with anti-SCG10 of extracts from ES-NSc cultured 3 days with EGF and bFGF, 3 days with PDGF, then 6 days without (left lane) or with IL6RIL6 added for the last 3, 6 or 9 days or throughout the 12 day culture as indicated. Mouse brain extract in the right lane. Reaction with anti-Erk as control. (B–C) ES-NSc (after 2 passages) cultured for 2 days with EGF and bFGF and 9 days without (B) or with IL6RIL6 for the last 3 days (C). Neurons stained for tubulin-βIII (green) are positive for SCG10 (red) as seen by yellow overlap (arrows in B) but with IL6RIL6 (C) many branched non-neuronal cells are SCG10 positive. (D–H) In another experiment with IL6RIL6 for the last 2 days, higher magnification shows that the $O4^+$ oligodendrocyte precursors (D) are also stained with anti-SCG10 (E) in the branches and in the cell body (see insert at reduced intensity). SCG10 stain (E) and overlap with O4 (F) reveal discrete accumulation of SCG10 before bifurcation of branches (arrow and line in E and F, compared to same in D). Such SCG10 accumulation sites are more numerous along branches of IL6RIL6 treated (H) than in untreated cells (G) where SCG10 is mainly in the cell body. (I–J) Silencing of SCG10 gene expression. ES-NSc cultured for 2 days with EGF and bFGF, then 2 days without, were transfected with control (I) or SCG10 siRNA (J) and after 1 day were treated for 5 days with IL6RIL6. A dense network of $O4^+$ (brown) cell branches and membrane sheets (arrow) developed in the control culture (I) but not if SCG10 is inhibited (J), the cells showing instead long straight processes (arrow). Neurons stained for tubulin-βIII (green). Size bars: B–C and I–J, 50 μm; D–H, 20 μm.

study of the incorporation and function of neurons or glial cells derived from fetal brain cell grafts (Shetty and Turner, 1999) or from ES cells (Scheffler et al., 2003). We adopted this transplantation paradigm as it allows quantitative comparison under similar conditions of donor cells obtained by different procedures or subjected to various growth conditions and pretreatments (Morrow et al., 2001; Scheffler et al., 2003).

For transplantations, we used as donor cells a fluorescent clone of Rosa11 ES cells (clone 19) prepared by transformation with EGFP plasmid. We compared the myelinating capacity of ES-NSc cultured 3 days with EGF and bFGF in the presence or absence of IL6RIL6 and then one more day without growth factors. At this time, the cultures treated with IL6RIL6 had $O4^+$ bipolar oligodendrocyte precursors that were slightly more elongated (Fig. 5B, arrows) than the control cultures (panel A). In both cases, 80–90% of the cells expressed EGFP and O4 (not shown). The cells, harvested by trypsinization, were deposited in fixed amounts on the surface of organotypic cultures of *shi/shi* mice brain slices, in the hippocampus region in most cases, and 2 weeks later serial thin-sections of the slices were examined after staining with anti-MBP antibodies. The treatment by IL6RIL6 produced a marked increase in myelinating capacity of the donor cells (Figs. 5C, G compared to H, I). Parallel tracks of MBP-stained fiber-like structures were observed in the shiverer brain

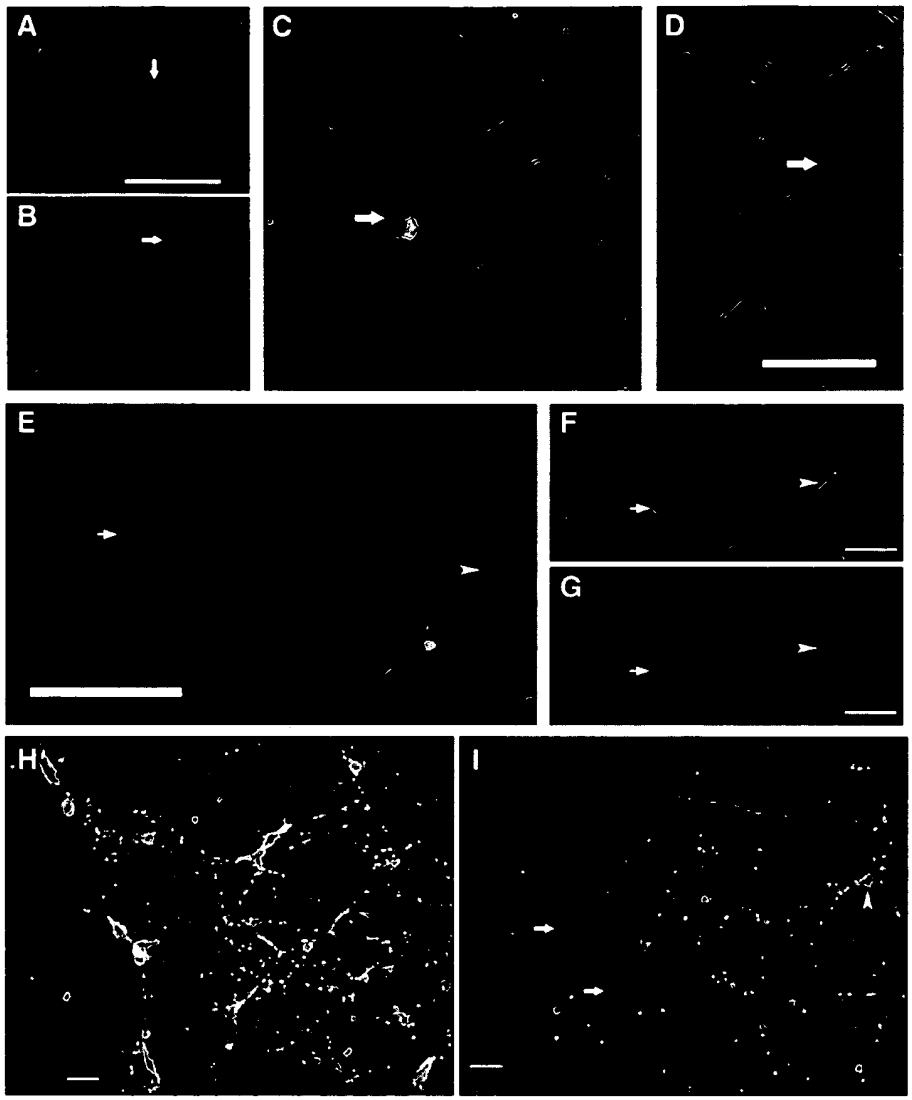

Fig. 4. Silencing of SCG10/Stmn2 expression affects oligodendrocytes morphological development. (A–D) ES-NSc cultured 2 days with EGF and bFGF, then 2 days without, were transfected with control (A, C) or SCG10 siRNA (B, D) and culture was continued 2 days without treatment (A, B) or with IL6RIL6 for the last day (C, D). With siSCG10, the $O4^-$ cells (green) have much less sub-branches and mainly a few straight processes. In panels C and D, additional staining for SCG10 shows (yellow) reduction of the protein in the cell body by SCG10 siRNA. Arrows point at cell bodies. (E) Fluorescent si*GLO* tracer of siRNA uptake (yellow) added to a transfection as in D (with IL6RIL6 and SCG10 siRNA) shows a transfected cell with typical SCG10 knock-down morphology (arrowhead), whereas in the same field, a cell that did not take up siRNA has the normal multi-branched phenotype (arrow). (F–G) ES-NSc transfected as above with SCG10 siRNA and 24 h later treated for 3 days with IL6RIL6. O4 staining (F, green) shows a multi-branched cell (arrow) next to a cell lacking sub-branching (arrowhead), correlating with the difference in SCG10 content (G, red). Cell nuclei stained with DAPI (blue). (H–I) Staining for O4 (green) and MBP (yellow) of ES-NSc transfected as above with control (H) or SCG10 siRNA (I) and 24 h later treated for 5 days with IL6RIL6. In I, arrowhead shows cell body and arrows point at membrane sheets. Size bars: 50 μm in all. Same magnification for A, B and for C, D.

tissue transplanted with IL6RIL6-treated cells (panel C). In this example, the position of the myelin tracks (rectangle of panel D, in the entorhinal cortex) was about 2.5 mm distant from the hippocampal structures where the donor cells were deposited, indicating migration of the myelinating cells within the brain slice. Some of the MBP-positive fibers in the track were several hundred microns long (C arrow) and cell bodies were visible adjacent to the fibers (line). Higher magnification (E) shows that nuclei of MBP-positive cells (lines) aligned with MBP-negative nuclei (white dots) of host intrafascicular oligodendrocytes, as typical in remyelination (Liu et al., 2000). EGFP fluorescence was present in MBP-positive cell bodies and processes indicating donor origin (F). Groups of MBP fibers were consistently seen in slices implanted with IL6RIL6-treated cells (as in C, G), whereas in slices implanted with cells not treated by IL6RIL6, there were fewer, shorter and thinner MBP fibers or sometimes only punctuated dots of MBP (panels H, I). The average number of myelinated fibers (of more than 10 μm length) visible per field was 34.0 ± 13.0 in the presence of IL6RIL6 versus 2.33 ± 3.8 in its absence (N = 10 fields each; $P < 0.0001$). In a similar experiment, MBP staining was quantitated by the mean integral optic density of color-specific pixels (Image-Pro Plus software) which showed that MBP staining was 4.6 fold higher with IL6RIL6 than without (N = 25 fields each; P = 0.03). The

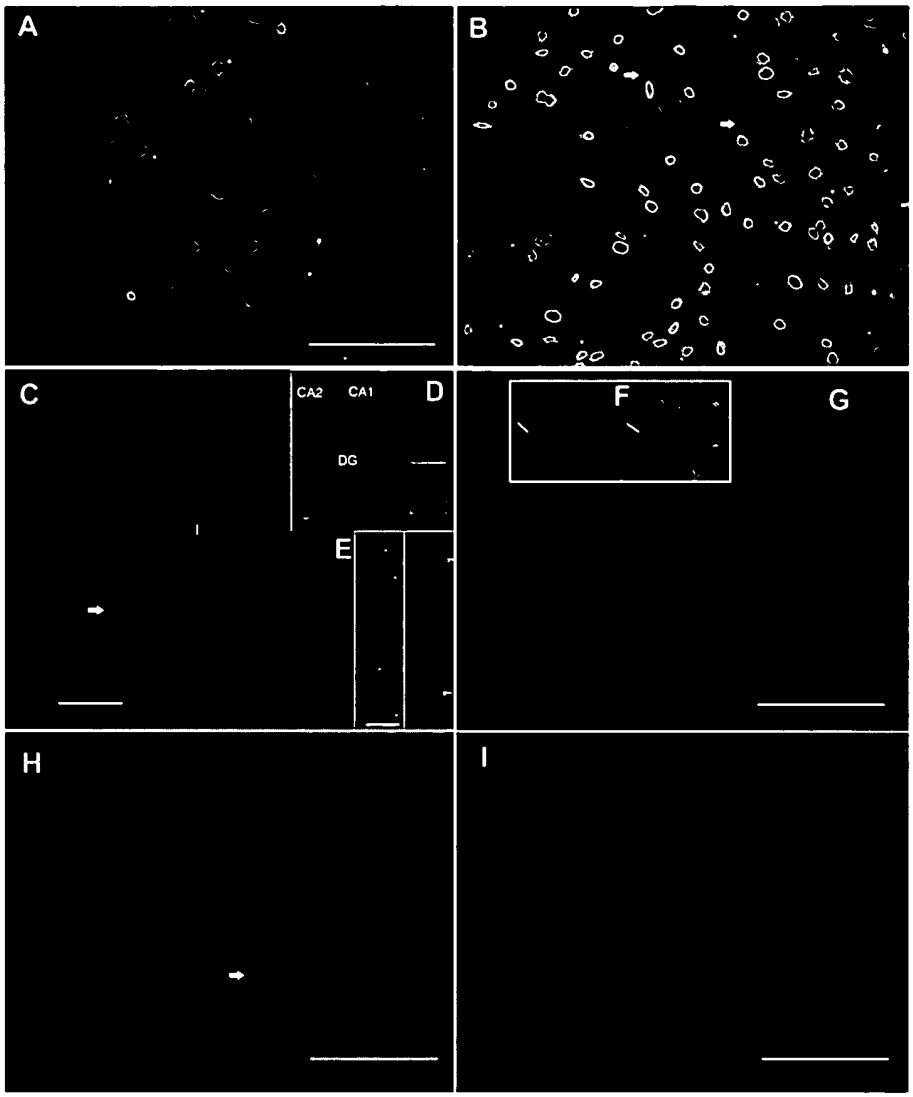

Fig. 5. Increased myelination capacity of IL6RIL6-treated oligodendrocyte precursors. (A, B) Prior to transplantation, parallel plates of ES-NSc (after 3 passages) were cultured 3 days with EGF and bFGF, then 1 day without growth factors (A) or same but with IL6RIL6 (B). Cells are $O4^+$ (red) and more elongated with IL6RIL6 treatment (arrows in B). Nuclei stained with DAPI (blue). (C–G) Cryostat sections (20 μm) of shiverer ($shi^{-/-}$) mice brain slices stained with anti-MBP antibodies (red) and DAPI (blue) on day 14 after having been transplanted with the IL6RIL6-treated cells show (C) dense arrays of long MBP^+ fibers (arrow points at one fiber). Location of this array at low magnification (rectangle in D) indicates it is in the entorhinal cortex about 2.5 mm from the hippocampus region where cells were deposited. High magnification shows MBP^+ cell bodies (lines in E right panel) aligned with intrafascicular nuclei (E, left panel, white dots). The MBP^+ cell bodies and processes show EGFP fluorescence (F, green) demonstrating donor cell origin. Arrays of MBP^+ fibers were seen in sections from other similarly transplanted brain slices (G). In contrast, shiverer mice brain slices transplanted with the cells not-treated by IL6RIL6 (as in A above) have only short and thin MBP^+ fibers (H, arrow) or dot-like MBP staining (I).Control non-transplanted slices from shi $^{-/-}$ mice had no MBP staining (not shown). Size bars: 100 μm except for E where bar is 20 μm. Labels in D are DG: dentate gyrus; CA1, CA2: pyramidal cell layers of hippocampus.

pretreatment of the donor ES-NSc by the gp130 activator therefore increases the extent of remyelinating fibers (number and length) in the dysmyelinated brain tissue.

Discussion

We describe a protocol for the improved differentiation and maturation of oligodendrocytes from murine ES cell-derived dissociated neurosphere cells (NSc) using the gp130 activator IL6RIL6. The ES-NSc, obtained by trypsinization of neurospheres, could be passaged repeatedly on polyornithine and stored frozen with most of the cells remaining nestin positive and multipotent to yield neurons, astrocytes and oligodendrocytes, in analogy to brain-derived neural stem cells (Reynolds and Weiss, 1996). Removal of growth factors (EGF, bFGF, PDGF) led to formation of small multipolar $O4^+$ oligodendrocyte precursors but addition of IL6RIL6 at various time during differentiation consistently led to a large increase in the size of the $O4^+$ cells with development of complex networks of long branches and formation of MBP-containing myelin membrane sheets. Compared to cells outgrowing from intact neurospheres (Zhang et al., 2004), the dissociated cells yield more homogenous populations with close to 40% $O4^+$ cells. Gene expression profiling confirmed the predominant NSc differentiation towards oligodendrocytes, since many myelin-related genes are among the most expressed genes in these cultures

(see Supplementary Tables 2, 3, online). Time course experiments and delayed addition of IL6RIL6 indicated that the marked change in oligodendrocyte morphology could occur after the cells had switched on transcription of myelin genes such as CNP and MBP. In particular, the IL6RIL6-induced formation of large MBP-containing membrane sheets, representing unfurled myelin sheaths such as seen in cultures of brain-derived mature oligodendrocytes (Carson et al., 1997), indicates that IL6RIL6 induces maturation of the ES cell-derived oligodendrocyte precursors.

Development of the oligodendrocyte long processes and branches involves microtubule extension over microfilament tracks (Carson et al., 1997; Song et al., 2001). Our finding that the microtubule-related gene SCG10/Stmn2 is among the most highly induced by IL6RIL6 is of interest, since this protein is known to inhibit tubulin polymerization and regulate microtubule dynamics in axons (Gavet et al., 1998; Grenningloh et al., 2004). We show here for the first time that SCG10 is present in oligodendrocyte branches. Moreover, inhibition of SCG10 gene expression by siRNA causes a profound alteration in the development of the network of branches, veins and peripheral accumulation of MBP, indicating that this stathmin-like protein is one of the mediators of IL6RIL6 effects on oligodendrocyte development. We confirmed the presence of SCG10 in oligodendrocytes from rat brain cortical cultures, and its increase upon IL6RIL6 treatment (not shown). The importance of microtubule dynamics and instability in the formation of oligodendrocyte branches has been stressed (Song et al., 2001) as well as the fact that microtubules, along which the MBP mRNA is transported, stop and do not enter in the regions forming the myelin membranes (Carson et al., 1997). The CNS myelin deficiency in the taiep rat mutant has been correlated with a defect in MBP mRNA transport resulting from a too dense packing of microtubules (Song et al., 2003). The microtubule depolymerization activity of Stmn2 could be part of the mechanism that maintains proper microtubule dynamics essential for development of the oligodendrocyte network of branches and its function for myelination. The observation that SCG10 tended to accumulate proximally to bifurcations of the branches further suggests that inhibition of tubulin polymerization may be part of the branching process. This could explain why SCG10 inhibition in IL6RIL6-treated cultures led to cells with long and straight processes. SCG10 is only one of the genes induced by IL6RIL6 and its role needs to be further studied together with the other changes in gene expression related to gp130 activation in the neurosphere cells (see Supplementary results, on line).

The gp130 activator IL6RIL6 has potential applications for transplantation since we show that it markedly increased the capacity of ES-NSc to myelinate brain tissue from dysmyelinated shiverer mice. To measure this capacity, we chose to transplant the cells into brain slices maintained in organotypic cultures because it allows to control the conditions and compare equal number of donor cells treated in different ways. As seen above, IL6RIL6 promotes terminal differentiation of ES-NSc in 9–12 days. However, the more differentiated oligodendrocytes seem to migrate much less from the implantation site than early precursors (Warrington et al., 1993; Yandava et al., 1999). Therefore, we used short-term IL6RIL6 treatments of the donor cells based on preliminary experiments which showed that after 3–4 days exposure of NSc to IL6RIL6 the cytokine could be removed without loss of the differentiative effect. As a result, early precursors pretreated by the gp130 activator could retain a better potential of further developing into mature myelinating cells within the brain tissue. Indeed, ES-NSc cultured for 4 days with IL6RIL6 treatment demonstrated after transplantation a much higher myelination capacity than untreated cells. After 2 weeks, there were long tracks of MBP-positive fibers, often with donor cell bodies (EGFP-labeled) adjacent to the fibers and aligned with host oligodendrocytes as typical in the CNS. Some of the tracks were over 500 μm long and their distance from the hippocampal site of deposition would indicate that the cells migrated 2–3 mm to areas of white matter. In the parallel cultures untreated by IL6RIL6, the MBP-positive fibers were much shorter and thinner, and the number of actual fibers was only about 7% of what was observed with the IL6RIL6 treatment.

The present protocol employing IL6RIL6 ex vivo may serve to prepare transplantable populations of ES-NSc with improved myelinating capacity. As a first measure of safety, it was verified that such ES-NSc injected intraventricularly to newborn mice were not tumorigenic after 2 months (Laffaire J., Harroch S., Chebath J, Revel M., unpublished). Injections of IL6RIL6 were previously shown to stimulate peripheral nerve myelination during sciatic nerve regeneration (Haggiag et al., 2001) in line with its effects on myelin protein Po and MBP gene transcription (Slutsky et al., 2003). IL6RIL6 also had promyelinating and neuroprotective effects when added to whole brain slices (Pizzi et al., 2004). Hence, IL6RIL6 could be applied ex vivo during the ES-NSc culture prior to transplantation to promote their myelination capacity, but the cytokine may also have further beneficial effects when administered in vivo after cell transplantation.

Experimental methods

Cell cultures

The murine ES cell line ROSA 11 was maintained as before (Zhang et al., 2004) on feeder layers of irradiated mouse embryo fibroblasts (MEF) in ES growth medium (DMEM with 15% fetal calf serum (FCS), 2 mM glutamine, 0.1 mM β-mercaptoethanol, 1% non-essential amino acid, 1% sodium pyruvate, LIF 1,000 u/ml) with daily medium change and splitting with 0.05% trypsin, 0.02% EDTA every 3 days. All cultures were incubated at 37°C in 5% CO_2. Prior to making embryoid bodies (EB), 6×10^5 ES cells were seeded in a 6 cm tissue culture plate over a layer of 3×10^5 irradiated MEF, in ES1 medium (DMEM/F12 with 20% serum replacement [InVitrogen, Carlsbad, CA], 2 mM glutamine, 0.1 mM β-mercaptoethanol, 2 μg/ml heparin) with 4 ng/ml of human recombinant bFGF (Sigma-Israel) with daily medium change. After 2–3 days, ES cells were detached from the MEF layer with 0.2 mg/ml dispase (InVitrogen) in phosphate buffered saline (PBS, Ca and Mg free) for 15 min at 37°C and clumps transferred to 9 cm bacterial dishes as suspension culture in ES1 medium lacking bFGF. After 4 days, the differentiating EBs were transferred to tissue culture dishes in EB defined medium (DMEM/F12, 25 μg/ml insulin, 100 μg/ml transferrin, 60 μM putrescine, 30 nM sodium selenite, 2 μg/ml heparin, 20 nM progesterone) with 20 ng/ml of bFGF, for 8–10 days with medium change every 2 days. Floating clumps, i.e. neurospheres (NS), were transferred to bacterial dishes in suspension in the same medium and used within 7 days.

About 100 NS were treated with 0.5 ml of 0.05% trypsin at 37°C for 10 min, then triturated 20 times with a 200 μl tip and trypsinization stopped with 0.1% BSA in EB defined medium. The dissociated ES-NSc were seeded on plates coated with a solution of 250 μg/ml fibronectin and 0.01% polyornithine (Sigma, St. Louis, MO) in serum-free N2 medium (DMEM/F12 with 1% N2 supplement [InVitrogen]) with EGF and bFGF (20 ng/ml each) for expansion 3–5 days. The cells were passaged by trypsinization or stored frozen. For differentiation, about 3×10^4 ES-NSc were plated on glass coverslips precoated with polyornithine, which were placed into 12-

well plates. The N2 medium was supplemented for the first 3 days with EGF and bFGF (10 ng/ml each) and with laminin 1 μg/ml (L2020, Sigma) to facilitate attachment and proliferation. After that, culture was in DMEM with 1% N2. Where indicated, PDGF-AA (20 ng/ml) was added for 3 days and then culture continued without it. The IL6RIL6 chimera (Chebath et al., 1997) was added at 100 ng/ml, starting at different times during the differentiation process as indicated (for a summary of the protocol, see Supplementary Table 4).

Gene expression knock-down for SCG10 was done with a pool of four double stranded siRNA sequences for mouse STMN2, accession number NM_025285, from Dharmacon (LaFayette, CO). Dissociated ES-NSc plated as above on polyornithin and fibronectin coated coverslips (3×10^4 cells/well in 12-well plates) were grown 2 days in DMEM/F12, 1% N2 with EGF and bFGF (20 ng/ml each) to 60% confluency, then 2 days in same without growth factors and transfected using siRNA and Dharmafect (Dharmacon) for 10 h on the cells. The transfection mixture was removed and culture continued in DMEM/F12, 1% N2 without or with IL6RIL6 100 ng/ml for 1 to 5 days. A pool of control siRNA was used as control. In some experiments, a fluorescent si*GLO* Risc-free tracer (Dharmacon) was added to the siRNA and showed at least 60% fluorescent cells in each transfection. For immunostaining, see below. Similar cultures in 6-well plates were used for extraction of RNA and proteins.

RNA and protein analysis

Total RNA was isolated using Tri-Reagent (Molecular Center, Cincinnati, OH) from ES-NSc cultured in 6-well plates for 9 days in conditions described in the text. Two series of RNA extracts were used to prepared cDNA and biotin-labeled cRNA for hybridization on four Mouse genome 430 2.0 arrays (Affymetrix, Santa Clara, CA), and fold induction analysis performed with MAS 5 statistical algorithms at the Crown Human Genome Center, Weizmann Institute of Science (Dr. Shirley Saban).

For immunoblots, proteins were extracted with RIPA buffer (50 mM Tris–HCl pH 7.4, 140 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1% protease inhibitor mix [Sigma-Israel], 1% (v/v) NP40 and 0.25% (w/v) Na Deoxycholate). Soluble proteins were analyzed on a 12% SDS-PAGE, and blotted onto nitrocellulose. The blot was reacted with anti-SCG10 antibodies (1:20,000) and with secondary HRP-labeled antibodies which were detected by ECL as described (Kamaraju et al., 2004).

Immunostaining

Cultures were fixed in 4% paraformaldehyde (PFA), washed and kept in PBS at 4°C. After blocking non-specific binding with 10% normal goat serum, oligodendrocytes were stained with anti-sulfatide O4 mouse monoclonal IgM antibodies (R&D system, MAB1326, 1:700) and Fluorescein-conjugated goat anti-mouse IgM (Chemicon International, Temecula, CA; 1:75). Staining for MBP was with rat monoclonal antibody ab7349 (Abcam, Cambridge, MA; 1:1000 dilution in 10% goat serum) followed by Cy3-conjugated Affipure goat anti-rat IgG (Jackson Immunoresearch Laboratories, West Grove, PA; 1:1000). Neurons were similarly stained with rabbit polyclonal anti-tubulin-βIII (Covance, Berkeley, CA, 1:200 in 1% goat serum) and astrocytes with Cy3-conjugated mouse monoclonal anti-GFAP (Sigma). Rabbit polyclonal anti-SCG10 (stathmin-like 2) C-terminal peptide (Gavet et al., 1998) was a kind gift from Dr. Andre Sobel (INSERM U706, Paris) and was used at the dilution 1:10,000 in 1% goat serum, followed by Cy3-conjugated goat anti rabbit (Jackson Immunoresearch). For double staining, cells were first stained for O4, fixed again with 3% PFA, blocked with 10% goat serum for 30 min, including 5 min of treatment with Triton-X100, and washed with PBS before reaction with the other antibody. For double staining with anti-SCG10, the mouse monoclonal antibody anti-tubulin-βIII (TuJ1, Covance; 1:400) and goat anti-mouse IgG conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR; 1:250) were used. Where shown, the nuclear fluorescent dye DAPI (Sigma; 0.05 μg/ml) was added last. All coverslips were mounted in Mowiol (Calbiochem, LaJolla, CA). An Olympus IX-70 FLA microscope with a DVC-1310C digital camera (DVC, Austin, TX) was used and images processed with Photoshop and analyzed with AlphaEase software (Alpha Innotech, San Leandro, CA).

Organotypic brain slice cultures, cell transplantation and myelin staining

C3Fe.SWV-*Mbp*^{shi}/J mice heterozygous for the MBP gene deletion (Roach et al., 1985) were obtained from Jackson Laboratory (Bar Harbor, Maine) and a colony of shiverer homozygous animals bred in our animal house. All procedures were according to the Weizmann Institute animal experiment committee guidelines. Vibroslicer VSLM1 (Campden Instruments, Sileby, UK) was used to prepare 400 μm-thick sagittal slices from the brain of 9-day old homozygous shiverer mice. About three slices were used per animal, representing the median part of the brain. Slices were maintained as interface cultures (Stoppini et al., 1991) on clear polyester membrane inserts 0.4 μm pore size (Transwell, Corning, Acton, MA; or Millicel, Millipore, Bedford, MA) in a 12-well plate. Wells were filled with 0.5 ml comprising 50% minimal essential medium (MEM with 25 mM HEPES, 4 mM $NaHCO_3$ and NaOH, pH 7.2), 25% Hank's solution, 25% horse serum, glucose 6.4 g/l, glutamine 2 mM, penicillin 100 U/ml and streptomycin 100 μg/ml. A third of the medium was replaced the first day and then every 2 days so as to finally arrive, within a week, to a serum-free slice medium (SFSM) of DMEM/F12 with 0.5% N2 supplement plus 1% B27 supplement (InVitrogen) with glutamine and antibiotics as above.

The ES cells Rosa 11 used for transplantation were first transfected with a plasmid for expression of the green fluorescent protein created from pEGFP-N1 (InVitrogen) by inserting its *Eco*RI/*Not*I fragment into the pEF-IRES puro plasmid. The pEF-EGFP-IRES-puro DNA was linearized with *Ase*I and 20 μg was electroporated into trypsinized Rosa 11 ES cells. Transfected cells were seeded (2×10^5 cells/well) in 6-well tissue culture plates coated with 0.1% gelatin and subjected to selection with puromycin 3 μg/ml in the ES growth medium. Fluorescent clones were tested for oligodendrocyte differentiation. EGFP$^+$ clone-19 ES cells were used to prepare neurospheres that were dissociated into NSc and passaged 3–4 times in tissue culture dishes coated with polyornithine and fibronectin in DMEM/F12, 1% N2, 20 ng/ml EGF and 20 ng/ml bFGF, as detailed above. Prior to transplantation, the EGF and bFGF concentrations were reduced to 10 ng/ml for 3 days, with or without addition of IL6RIL6 100 ng/ml, and culture was continued for 1 day after removal of growth factors. Following trypsinization, an aliquot of the cells was plated on polyornithine-coated coverslips in DMEM/F12, 1%N2 for verifying O4 staining and viability. About 2×10^4 cells, in 0.5 μl of culture medium, were deposited onto the brain slices (precultured 10 days as above), in the hippocampus region for most experiments. After 1 h in the incubator, another 100 μl of culture medium was added on the slice surface, and the plates incubated for another 14 days with SFSM medium change every other days. The slices were fixed in 4% paraformaldehyde at 4°C for 2–3 h and cryoprotected in phosphate-buffered 30% sucrose overnight. Serial 25 μm-thick sagittal cryostat sections were prepared from each slice, mounted on superfrost plus slides (Erie Scientific, Portsmouth, NH) and stored frozen until staining with anti-MBP antibodies as above. Sections were also examined for EGFP fluorescence and DAPI nuclear staining. The Image-ProPlus software(Media Cybernetics, Silver Spring, MD) was used to measure the Integral Optic Density (IOD) of color-specific pixels.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mcn.2005.10.014.

References

Asou, H., Hamada, K., Miyazaki, T., Sakota, T., Hayashi, K., Takeda, Y., Marret, S., Delpech, B., Itoh, K., Uyemura, K., 1995. CNS myelinogenesis in vitro: time course and pattern of rat oligodendrocyte development. J. Neurosci. Res. 40, 519–534.

Barres, B.A., Schmid, R., Sendnter, M., Raff, M.C., 1993. Multiple extracellular signals are required for long-term oligodendrocyte survival. Development 118, 283–295.

Barres, B.A., Lazar, M.A., Raff, M.C., 1994a. A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development. Development 120, 1097–1108.

Barres, B.A., Raff, M.C., Gaese, F., Bartke, I., Dechant, G., Barde, Y.A., 1994b. A crucial role for neurotrophin-3 in oligodendrocyte development. Nature 367, 371–375.

Barres, B.A., Burne, J.F., Holtmann, B., Thoenen, H., Sendtner, M., Raff, M.C., 1996. Ciliary neurotrophic factor enhances the rate of oligodendrocyte generation. Mol. Cell. Neurosci. 8, 146–156.

Bogler, O., Wren, D., Barnett, S.C., Land, H., Noble, M., 1990. Cooperation between two growth factors promotes extended self-renewal and inhibits differentiation of oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells. Proc. Natl. Acad. Sci. U. S. A. 87, 6368–6372.

Brustle, O., Jones, K.N., Learish, R.D., Karram, K., Choudhary, K., Wiestler, O.D., Duncan, I.D., McKay, R.D., 1999. Embryonic stem cell-derived glial precursors: a source of myelinating transplants. Science 285, 754–756.

Campagnoni, A.T., Verdi, J.M., Verity, A.N., Amur-Umarjee, S., Byravan, S., 1991. Posttranscriptional regulation of myelin protein gene expression. Ann. N. Y. Acad. Sci. 633, 178–188.

Cao, Q., Benton, R.L., Whittemore, S.R., 2002. Stem cell repair of central nervous system injury. J. Neurosci. Res. 68, 501–510.

Carson, J.H., Worboys, K., Ainger, K., Barbarese, E., 1997. Translocation of myelin basic protein mRNA in oligodendrocytes requires microtubules and kinesin. Cell Motil. Cytoskelet. 38, 318–328.

Chebath, J., Fischer, D., Kumar, A., Oh, J.W., Kolett, O., Lapidot, T., Fischer, M., Rose-John, S., Nagler, A., Slavin, S., Revel, M., 1997. Interleukin-6 receptor–interleukin-6 fusion proteins with enhanced interleukin-6 type pleiotropic activities. Eur. Cytokine Netw. 8, 359–365.

Fischer, M., Goldschmitt, J., Peschel, C., Brakenhoff, J.P., Kallen, K.J., Wollmer, A., Grotzinger, J., Rose-John, S., 1997. A bioactive designer cytokine for human hematopoietic progenitor cell expansion. Nat. Biotechnol. 15, 142–145.

Foster, L.M., Landry, C., Phan, T., Campagnoni, A.T., 1995. Conditionally immortalized oligodendrocyte cell lines migrate to different brain regions and elaborate 'myelin-like' membranes after transplantation into neonatal shiverer mouse brains. Dev. Neurosci. 17, 160–170.

Gard, A.L., Pfeiffer, S.E., 1993. Glial cell mitogens bFGF and PDGF differentially regulate development of O4+GalC− oligodendrocyte progenitors. Dev. Biol. 159, 618–630.

Gard, A.L., Williams, W.C., Burrell, M.R., 1995. Oligodendroblasts distinguished from O-2A glial progenitors by surface phenotype (O4+GalC−) and response to cytokines using signal transducer LIFR beta. Dev. Biol. 167, 596–608.

Gavet, O., Ozon, S., Manceau, V., Lawler, S., Curmi, P., Sobel, A., 1998. The stathmin phosphoprotein family: intracellular localization and effects on the microtubule network. J. Cell Sci. 111 (Pt. 22), 3333–3346.

Glaser, T., Perez-Bouza, A., Klein, K., Brustle, O., 2005. Generation of purified oligodendrocyte progenitors from embryonic stem cells. FASEB J. 19, 112–114.

Grenningloh, G., Soehrman, S., Bondallaz, P., Ruchti, E., Cadas, H., 2004. Role of the microtubule destabilizing proteins SCG10 and stathmin in neuronal growth. J. Neurobiol. 58, 60–69.

Gumpel, M., Gout, O., Lubetzki, C., Gansmuller, A., Baumann, N., 1989. Myelination and remyelination in the central nervous system by transplanted oligodendrocytes using the shiverer model. Discussion on the remyelinating cell population in adult mammals. Dev. Neurosci. 11, 132–139.

Haggiag, S., Zhang, P.L., Slutzky, G., Shinder, V., Kumar, A., Chebath, J., Revel, M., 2001. Stimulation of myelin gene expression in vitro and of sciatic nerve remyelination by interleukin-6 receptor–interleukin-6 chimera. J. Neurosci. Res. 64, 564–574.

Heinrich, M., Gorath, M., Richter-Landsberg, C., 1999. Neurotrophin-3 (NT-3) modulates early differentiation of oligodendrocytes in rat brain cortical cultures. Glia 28, 244–255.

Kamaraju, A.K., Adjalley, S., Zhang, P., Chebath, J., Revel, M., 2004. C/EBP-delta induction by gp130 signaling. Role in transition to myelin gene expressing phenotype in a melanoma cell line model. J. Biol. Chem. 279, 3852–3861.

Knapp, P.E., Bartlett, W.P., Skoff, R.P., 1987. Cultured oligodendrocytes mimic in vivo phenotypic characteristics: cell shape, expression of myelin-specific antigens, and membrane production. Dev. Biol. 120, 356–365.

Kollet, O., Aviram, R., Chebath, J., ben-Hur, H., Nagler, A., Shultz, L., Revel, M., Lapidot, T., 1999. The soluble interleukin-6 (IL-6) receptor/IL-6 fusion protein enhances in vitro maintenance and proliferation of human CD34(+)CD38(−/low) cells capable of repopulating severe combined immunodeficiency mice. Blood 94, 923–931.

Lachapelle, F., Gumpel, M., Baulac, M., Jacque, C., Duc, P., Baumann, N., 1983. Transplantation of CNS fragments into the brain of shiverer mutant mice: extensive myelination by implanted oligodendrocytes. I. Immunohistochemical studies. Dev. Neurosci. 6, 325–334.

Lillien, L.E., Sendtner, M., Raff, M.C., 1990. Extracellular matrix-associated molecules collaborate with ciliary neurotrophic factor to induce type-2 astrocyte development. J. Cell Biol. 111, 635–644.

Liu, S., Qu, Y., Stewart, T.J., Howard, M.J., Chakrabortty, S., Holekamp, T.F., McDonald, J.W., 2000. Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. Proc. Natl. Acad. Sci. U. S. A. 97, 6126–6131.

MacDonald, S.C., Simcoff, R., Jordan, L.M., Dodd, J.G., Cheng, K.W., Hochman, S., 2002. A population of oligodendrocytes derived from multipotent neural precursor cells expresses a cholinergic phenotype in culture and responds to ciliary neurotrophic factor. J. Neurosci. Res. 68, 255–264.

Marmur, R., Kessler, J.A., Zhu, G., Gokhan, S., Mehler, M.F., 1998. Differentiation of oligodendroglial progenitors derived from cortical multipotent cells requires extrinsic signals including activation of gp130/LIFbeta receptors. J. Neurosci. 18, 9800–9811.

Mayer, M., Bhakoo, K., Noble, M., 1994. Ciliary neurotrophic factor and leukemia inhibitory factor promote the generation, maturation and survival of oligodendrocytes in vitro. Development 120, 143–153.

McDonald, J.W., Liu, X.Z., Qu, Y., Liu, S., Mickey, S.K., Turetsky, D., Gottlieb, D.I., Choi, D.W., 1999. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. Nat. Med. 5, 1410–1412.

McKay, R., 1997. Stem cells in the central nervous system. Science 276, 66–71.

Morrow, T., Song, M.R., Ghosh, A., 2001. Sequential specification of neurons and glia by developmentally regulated extracellular factors. Development 128, 3585–3594.

Nistor, G.I., Totoiu, M.O., Haque, N., Carpenter, M.K., Keirstead, H.S., 2005. Human embryonic stem cells differentiate into oligodendrocytes in high purity and myelinate after spinal cord transplantation. Glia 49, 385–396.

Pfeiffer, S.E., Warrington, A.E., Bansal, R., 1993. The oligodendrocyte and its many cellular processes. Trends Cell Biol. 3, 191–197.

Pizzi, M., Sarnico, I., Boroni, F., Benarese, M., Dreano, M., Garotta, G., Valerio, A., Spano, P., 2004. Prevention of neuron and oligodendrocyte degeneration by interleukin-6 (IL-6) and IL-6 receptor/IL-6 fusion protein in organotypic hippocampal slices. Mol. Cell. Neurosci. 25, 301–311.

Reubinoff, B.E., Itsykson, P., Turetsky, T., Pera, M.F., Reinhartz, E., Itzik, A., Ben-Hur, T., 2001. Neural progenitors from human embryonic stem cells. Nat. Biotechnol. 19, 1134–1140.

Reynolds, B.A., Weiss, S., 1996. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev. Biol. 175, 1–13.

Roach, A., Takahashi, N., Pravtcheva, D., Ruddle, F., Hood, L., 1985. Chromosomal mapping of mouse myelin basic protein gene and structure and transcription of the partially deleted gene in shiverer mutant mice. Cell 42, 149–155.

Rogister, B., Ben-Hur, T., Dubois-Dalcq, M., 1999. From neural stem cells to myelinating oligodendrocytes. Mol. Cell. Neurosci. 14, 287–300.

Rubio, N., Rodriguez, R., Arevalo, M.A., 2004. In vitro myelination by oligodendrocyte precursor cells transfected with the neurotrophin-3 gene. Glia 47, 78–87.

Scheffler, B., Schmandt, T., Schroder, W., Steinfarz, B., Husseini, L., Wellmer, J., Seifert, G., Karram, K., Beck, H., Blumcke, I., Wiestler, O.D., Steinhauser, C., Brustle, O., 2003. Functional network integration of embryonic stem cell-derived astrocytes in hippocampal slice cultures. Development 130, 5533–5541.

Shetty, A.K., Turner, D.A., 1999. Neurite outgrowth from progeny of epidermal growth factor-responsive hippocampal stem cells is significantly less robust than from fetal hippocampal cells following grafting onto organotypic hippocampal slice cultures: effect of brain-derived neurotrophic factor. J. Neurobiol. 38, 391–413.

Slutsky, S.G., Kamaraju, A.K., Levy, A.M., Chebath, J., Revel, M., 2003. Activation of myelin genes during transdifferentiation from melanoma to glial cell phenotype. J. Biol. Chem. 278, 8960–8968.

Song, J., Goetz, B.D., Baas, P.W., Duncan, I.D., 2001. Cytoskeletal reorganization during the formation of oligodendrocyte processes and branches. Mol. Cell. Neurosci. 17, 624–636.

Song, J., Carson, J.H., Barbarese, E., Li, F.Y., Duncan, I.D., 2003. RNA transport in oligodendrocytes from the taiep mutant rat. Mol. Cell. Neurosci. 24, 926–938.

Stoppini, L., Buchs, P.A., Muller, D., 1991. A simple method for organotypic cultures of nervous tissue. J. Neurosci. Methods 37, 173–182.

Taga, T., Kishimoto, T., 1997. gp130 and the interleukin-6 family of cytokines. Annu. Rev. Immunol. 15, 797–819.

Trapp, B.D., Moench, T., Pulley, M., Barbosa, E., Tennekoon, G., Griffin, J., 1987. Spatial segregation of mRNA encoding myelin-specific proteins. Proc. Natl. Acad. Sci. U. S. A. 84, 7773–7777.

Valerio, A., Ferrario, M., Dreano, M., Garotta, G., Spano, P., Pizzi, M., 2002. Soluble interleukin-6 (IL-6) receptor/IL-6 fusion protein enhances in vitro differentiation of purified rat oligodendroglial lineage cells. Mol. Cell. Neurosci. 21, 602–615.

Verity, A.N., Campagnoni, A.T., 1988. Regional expression of myelin protein genes in the developing mouse brain: in situ hybridization studies. J. Neurosci. Res. 21, 238–248.

Vescovi, A.L., Reynolds, B.A., Fraser, D.D., Weiss, S., 1993. bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. Neuron 11, 951–966.

Warrington, A.E., Pfeiffer, S.E., 1992. Proliferation and differentiation of O4+ oligodendrocytes in postnatal rat cerebellum: analysis in unfixed tissue slices using anti-glycolipid antibodies. J. Neurosci. Res. 33, 338–353.

Warrington, A.E., Barbarese, E., Pfeiffer, S.E., 1993. Differential myelinogenic capacity of specific developmental stages of the oligodendrocyte lineage upon transplantation into hypomyelinating hosts. J. Neurosci. Res. 34, 1–13.

Yandava, B.D., Billinghurst, L.L., Snyder, E.Y., 1999. "Global" cell replacement is feasible via neural stem cell transplantation: evidence from the dysmyelinated shiverer mouse brain. Proc. Natl. Acad. Sci. U. S. A. 96, 7029–7034.

Zhang, S.C., Wernig, M., Duncan, I.D., Brustle, O., Thomson, J.A., 2001. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat. Biotechnol. 19, 1129–1133.

Zhang, P., Chebath, J., Lonai, P., Revel, M., 2004. Enhancement of oligodendrocyte differentiation from murine embryonic stem cells by an activator of gp130 signaling. Stem Cells 22, 344–354.